This thesis is posthumously dedicated to

Vincent Patrick and Frances Marie Carroll.

THE PLANT CONTRIBUTION TO THE SOYBEAN-*RHIZOBIUM* SYMBIOSIS.

by

Bernard John Carroll

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Doctor of Philosophy

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DECLARATION

The research described in this thesis is my own work, except where acknowledgement is made, and has not been submitted for any other degree. The planning and execution of experiments described in Chapters 4, 5, 6 and 10 were done in collaboration with Dr. David McNeil. My contribution to the research for these chapters was 50%, 60%, 63% and 70%. I processed the data and interpreted the results for these chapters.

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ABSTRACT

The focus of this investigation was on the regulation of nodulation and nitrogen fixation in soybean. Major emphasis was given to the inhibitory influence of nitrate on the soybean-Rhizobium japonicum symbiosis. Several nodulation and nitrate metabolism mutants of soybean were isolated.

Fifteen independent nitrate-tolerant symbiotic (nts) mutants were obtained from 2,500 M_2 families. In the presence of exogenous nitrate, nodule number, nodule mass and nitrogenase activity were several-fold those of the wild type cultured under identical conditions. Mutant nts382 was characterized in the greatest detail. This mutant nodulated significantly more than the parent cultivar Bragg in the presence or absence of several combined nitrogen sources (KNO_3, urea, NH_4Cl and NH_4NO_3). The number and density of nodules on the tap root and lateral roots was considerably increased in nts382. Furthermore, nitrate stimulated growth in both the wild type and nts382, and these lines had similar nitrate reductase activity. These results indicate that nts382 is affected in a nodule-development regulatory gene and not in a gene related to nitrate assimilation. Although nts382 is a supernodulator, inoculation with R. japonicum was necessary to induce nodule formation and both trial strains CB1809 (=USDA136) and USDA110 elicited the mutant phenotype. Mutant nts382 plants were generally smaller than wild-type plants and specific nitrogenase activity (activity per unit of nodule mass) was lower in the mutant. The mode of inheritance of the mutant character was investigated in four of the nts lines, and examples of both recessive and incompletely dominant inheritance was observed. The nts mutants are discussed in the context of regulation

of nodulation and of hypotheses that have been proposed to explain nitrate inhibition of nodulation. In addition to the nts nodulation mutants, three non-nodulating soybean mutants were also isolated.

Two constitutive nitrate reductase (cNR)-deficient mutants, NR328 and NR345, were recovered from an in vivo screen for a cNR activity. NR345 is similar to the previously isolated mutant nr_1 (Nelson et al. 1983; Harper et al., 1985), but NR328 is different. All of these mutants possess inducible nitrate reductase activity (Nelson et al., 1983; this study) and nodulation in these lines was inhibited by nitrate. However, NR328 is unique in that it did have a low level of cNR activity and, also, it eventually developed necrosis of the leaf margins when grown on nitrate.

Physiological studies on the wild-type parent cultivar Bragg indicated that oxygen supply to the nodule was a major factor limiting nitrogenase activity in nitrate- and dark-treated plants. There was no evidence of bacteroid carbohydrate deprivation during nitrate-induced nodule senescence, but there was evidence of this in the latter stages of dark-induced nodule senescence. These results are discussed in view of hypotheses that have been proposed to explain nitrate inhibition of nitrogenase activity in legume root nodule symbioses.

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CHAPTER ONE

GENERAL INTRODUCTION

Nitrogen is a fundamental constituent of proteins and nucleic acids. Its importance is emphasized by the fact that it is often the most limiting element in agriculture (Beringer, 1984). There is, of course, an abundance of molecular dinitrogen (N_2) in the earth's atmosphere, but only a limited number of prokaryotes are capable of reducing N_2 to ammonium, which can be assimilated into organic molecules. This process is called biological nitrogen fixation and the reaction is catalysed by the prokaryote-encoded enzyme nitrogenase (Bergersen, 1982).

Terrestrial plants absorb combined nitrogen from soil reserves, usually in the form of nitrate (Beringer, 1984). Legumes, however, have an additional means of obtaining nitrogen through a symbiotic association with diazotrophic soil bacteria of the genus *Rhizobium*. In general, a particular legume species forms a symbiosis with a specific *Rhizobium* species (Vincent, 1974). The structural entity of these symbioses is the root nodule, which harbours the nitrogen-fixing endophyte. In elementary terms, the legume host contributes energy derived from photosynthesis and, in return, the *Rhizobium* bacteroids within the nodule supply the host with ammonium from nitrogen fixation (Bergersen, 1982).

The magnitude of legume-*Rhizobium* symbioses is substantial in agricultural terms and it can be as high as several hundred kg of nitrogen per hectare per annum (Vincent, 1974; Beringer, 1984). However, the extent of symbiosis is tightly regulated by internal control mechanisms in the host and by environmental conditions. In the subsequent sections describing the root nodule symbiosis in more detail, emphasis is given to the regulation of nodulation and nitrogen fixation in soybean.

1.1 The Sequence of Events in Nodule Formation

The phenotypic sequence of events in nodule formation has been described by Vincent (1980) and is listed in Table 1.1.

1.1.1 Pre-infection events in nodule formation

The first clearly defined pre-infection event is rhizobial colonization of the rhizosphere (Broughton, 1978), followed by attachment of *Rhizobium* to the root surface. As alluded to earlier, there is a high degree of specificity in legume-*Rhizobium* symbioses and *R. japonicum*, for example, infects and nodulates soybean but not any of the temperate legumes. The lectin-recognition hypothesis has been put forward to explain the mechanism of host-*Rhizobium* specificity (Bohlool and Schmidt, 1974; Dazzo and Hollingsworth, 1984). In essence, this hypothesis states that specificity occurs at the point of attachment and that a host-encoded lectin on the root surface mediates attachment by binding to unique carbohydrate groups on the *Rhizobium* surface. However, some critical remarks have been made on this proposal by Robertson *et al.* (1981). Specificity in legume-*Rhizobium* symbioses is not restricted to attachment of *Rhizobium* to the roots and may occur at other stages of nodulation (Bauer, 1981). Furthermore, *Rhizobium* strains that are capable of binding to a legume lectin are not necessarily able to nodule the respective legume species (Wong, 1980; Stacey *et al.*, 1984).

In view of this controversy, some pertinent information has been obtained for the soybean - *R. japonicum* symbiosis. Stacey *et al.* (1982, 1984) have identified a non-nodulating *R. japonicum* mutant that fails to bind to soybean roots. However, the mutant binds to soybean lectin to the same extent as the wild-type parent strain does (Stacey *et al.*, 1984). Thus, soybean lectin is not the direct and

Table 1.1: The sequence of events in legume-*Rhizobium* symbioses as proposed by Vincent (1980).

Table 1.1:

I	PREINFECTION		
1.	Multiplication on root surface ('rhizoplane)	Root colonisation	Roc
2.	Attachment to root surface	Root adhesion	Roa
3.	Branching of root-hairs	Hair branching	Hab
4.	'Marked' curling of root-hairs	Hair curling	Hac
II	INFECTION AND NODULE FORMATION		
5.	Formation of infection thread	Infection	Inf
6.	Development of nodule meristem; nodule development and differentiation	Nodule initiation	Noi
7.	'Intracellular' release of *Rhizobium* from infection thread	Bacterial release	Bar
8.	'Intracellular' multiplication of *Rhizobium* and development of full bacteroid form	Bacteroid development	Bad
III	NODULE FUNCTION		
9.	Reduction of $N_2 \rightarrow NH_4^+$ (Nitrogenase)	Nitrogen fixation	Nif
10.	Complementary bio chemical and physiological functions	Complementary functions	Cof
11.	Persistence of nodule function	Nodule persistence	Nop

exclusive mediator of *R. japonicum* attachment to soybean roots. They suggested that components of the *R. japonicum* cell wall, other than those involved in lectin binding, may be mediating attachment between the macro- and microsymbiont. Consistent with this hypothesis, Vesper and Bauer (1984) have provided evidence that pili produced by *R. japonicum* are the primary mediators of attachment. Antiserum produced against these pili inhibited rhizobial attachment to the root and nodulation (Vesper and Bauer, 1984).

Lectins may, however, have an alternative role in the soybean root nodule symbiosis (Halverson and Stacey, 1984, 1985). The possible involvement of host-encoded lectin was demonstrated in studies using a slow-to-nodulate *R. japonicum* mutant, characterized by delayed nodulation (Stacey *et al.*, 1982). The mutant strain promptly nodulated soybeans, provided it was pre-incubated in soybean root exudate prior to inoculation (Halverson and Stacey, 1984). The active ingredient in the root exudate was shown to be a lectin (Halverson and Stacey, 1985). Thus, host lectins may be involved in conditioning the *Rhizobium* for nodulation.

1.1.2 Infection thread formation and nodule initiation

Rhizobium invade the root systems of legumes via infection threads (Dart, 1974; Rolfe and Shine, 1984), and it has been assumed that infection thread formation precedes nodule initiation (Vincent, 1980; Table 1.1). However, Calvert *et al.* (1984a) have shown that nodule initiation does not require infection thread formation in soybean. Furthermore, a high proportion of infection loci in soybean are pseudo-infections; that is, confined areas of cortical cell divisions without a corresponding infection thread (Calvert *et al.*, 1984b).

In soybean, infection threads are normally located in root hair cells, but less frequently may also be initiated in epidermal cells devoid of root hairs (Bauer, 1981). Recent studies have shown that soybean root hair infection always occurs in the markedly curled region of the root hair (Turgeon and Bauer, 1984). Furthermore, mature root hairs are generally not receptive to *R. japonicum* invasion (Calvert *et al.*, 1984b) and nodules usually form in the region of the root that is close to the root tip at the time of inoculation (Bauer, 1981). Infection threads often occur in close proximity to one another (Roughley *et al.*, 1970; Calvert *et al.*, 1984b) and it is therefore interesting to note that Gulash *et al.* (1984) recently observed that clouds of *R. meliloti* were attached to localized sites on the surface of the infectible region of *Medicago sativa* roots. Perhaps this phenomenon plays a role in the infection process (Gulash *et al.*, 1984).

Under conditions conducive to nodulation, the number of nodules formed is tightly regulated and is considerably less than the number of infection threads (Roughley *et al.*, 1970; Bauer, 1981). Both mature nodules (Nutman, 1952) and early infections (Pierce and Bauer, 1983; Kosslak and Bohlool, 1984) appear to be involved in curbing the success of infections that are subsequently formed. This will be discussed in more detail in Chapter 7.

1.2 Nodule Functioning

The reduction of N_2 requires considerable energy (Bergersen, 1982). In addition to supplying the bacteroids with energy-yielding substrates, provisions must be made in the nodule for adequate O_2 and N_2 supplies (Sinclair and Goudriaan, 1981) and also for the assimilation of symbiotically-fixed nitrogen (Miflin and Cullimore, 1984). The host cytoplasm must also supply other minor, but essential,

nutrients to the microsymbiont (Verma and Nadler, 1984). All of these processes are potential candidates for regulating the level of nitrogenase activity in the nodule.

1.2.1 Carbohydrate metabolism in the nodule

The dependence of nitrogen fixation on energy supply is reflected in the positive correlation between photosynthesis and nodule activity. Generally, environmental conditions that stimulate photosynthesis also stimulate nitrogen fixation (Hardy and Havelka, 1976). Sucrose is the major carbohydrate transported from soybean leaves to the nodules (Reibach and Streeter, 1983), where it is metabolized to form many species of carbohydrate (Streeter and Bosler, 1976). It is not clear at this stage which carbon source(s) is used by the bacteroids to support nitrogenase activity (Reibach and Streeter, 1983; La Rue *et al.*, 1984). However, it does appear that bacteroids must be able to utilize succinate or another C_4-dicarboxylate to be fully efficient in nitrogen fixation. This statement is based on the observations that succinate uptake-deficient mutants of *R. trifolii* (Ronson *et al.*, 1981) and *R. leguminosarum* (Glenn and Brewin, 1981) form ineffective nodules on the respective host species. Soybean bacteroids vigorously oxidize succinate (Stovall and Cole, 1978; McNeil, Carroll and Gresshoff, unpublished data), but it is possible that other substrates play a role in supporting bacteroid nitrogenase activity in the nodule. This will be discussed in greater detail in Chapter 4.

Hydrogen (H_2) and carbon dioxide (CO_2) are major by-products of bacteroid nitrogen fixation (Bergersen, 1982). H_2 evolution is an intrinsic property of active nitrogenase and the energy loss through

this avenue is considered to be at least 25% of the total used by the nitrogenase complex (Brewin, 1984). Some *Rhizobium* strains have an uptake hydrogenase which prevents or at least curbs energy loss through H_2 evolution (Brewin, 1984). Although the uptake hydrogenase enzyme is coded for by the *Rhizobium* genome, the host genotype can affect the extent of H_2 evolution from nodules (Keyser *et al.*, 1982; Bedmar and Phillips, 1984).

Carbon skeletons are required for the assimilation of nitrogen as well as for supporting respiration. CO_2 fixation occurs in nodules via PEP carboxylase and a major role for this process is to generate carbon compounds required for assimilation and transport of symbiotically-fixed nitrogen (Vance *et al.*, 1983; Maxwell *et al.*, 1984).

1.2.2 Nitrogen assimilation in the nodule.

Host-encoded enzymes are considered to be responsible for the assimilation of ammonium produced by the bacteroids (Miflin and Cullimore, 1984). The tropical legumes, including soybean, generally export assimilated nitrogen out of the nodule in the form of ureides, whereas many of the temperate legumes translocate amides from the nodule (Sprent, 1980). In soybean, the pathway of ureide synthesis has been studied as has the location of many of the ureide-related enzymes (Boland *et al.*, 1982; Hanks *et al.*, 1983).

1.2.3 O_2 supply in the nodule.

Whilst bacteroids and the host cytoplasm require O_2 for respiration, the nitrogenase enzyme complex is acutely sensitive to inactivation by O_2. This is the paradox regarding O_2 supply to the nitrogen-fixing region of the nodule (Bergersen, 1982).

The requirement for precise delivery of O_2 into the nodule is satisfied by the cooperative effects of structural protection (Sinclair and Goudriaan, 1981) and leghemoglobin-mediated O_2 transport (Appleby, 1984). Substantial gradients in O_2 concentrations have been measured across the soybean nodule cortex (Tjepkema and Yocum, 1974). Sinclair and Goudriaan (1981) deduced from a mathematical model that an O_2 diffusion barrier in the nodule cortex was necessary to prevent inactivation of nitrogenase. They also postulated that this diffusion barrier would not substantially inhibit the entry of N_2 into the nodule. The other important factor in stabilizing a low O_2 concentration around the bacteroids is the presence of leghemoglobin in the infected cells of the nodule (Verma and Nadler, 1984). The function of leghemoglobin has been comprehensively reviewed by Appleby (1984). The O_2 diffusion barrier and the apo-protein of leghemoglobin are plant contributions, whereas the protoheme moiety of leghemoglobin is considered to be a product of *Rhizobium* (Appleby, 1984).

There is evidence that the O_2 delivery system in legume nodules is not rigid and that it is subject to regulation (Criswell *et al.*, 1976, 1977; Witty *et al.*, 1984). This possibility will be addressed in more detail in Chapter 5.

1.3 Soil Nitrate: A Major Factor Regulating Nodulation and Nitrogen Fixation in Legumes

Several environmental factors affect the extent of symbiosis in legumes (Lie, 1974). These include temperature, moisture, O_2 supply, atmospheric CO_2 levels, light (photosynthetic and non-photosynthetic), pH, salinity and other soil factors. However, under

optimal field conditions, soil nitrate is considered to be a principle factor regulating the extent of nodulation and nitrogen fixation (Harper, 1976). Small amounts of nitrate stimulate the symbiosis (Koermendy and Eaglesham, 1984), whereas high nitrate levels decrease the contribution of symbiotically-fixed nitrogen to total crop nitrogen (Herridge, 1982) without increasing yield (Harper, 1974; McNeil and La Rue, 1984). In terms of total combined nitrogen present in a legume-containing cropping system, nitrate inhibition may be agronomically unfavourable. Inhibitory nitrate levels adversely affect all the stages in the symbiosis (Carroll and Gresshoff, 1983), from binding of *Rhizobium* to root hairs (Dazzo *et al.*, 1981) through to nodule senescence (Chen and Phillips, 1977).

Both dinitrogen and nitrate reduction are energy expensive processes (Beringer, 1984). Normally, growth conditions which increase photosynthesis also increase nitrogen fixation (Hardy and Havelka, 1976) and nitrate reduction (Nicholas *et al.*, 1976a, b). The carbohydrate deprivation hypothesis has been put forward to explain nitrate inhibition and argues that nitrate assimilation outcompetes symbiotic processes for available photosynthate (Oghoghorie and Pate, 1971; Gibson and Pagan, 1977). Consistent with this hypothesis, the presence of nitrate in the growth medium curbs the translocation of photosynthate into nodules (Small and Leonard, 1969; Latimore *et al.*, 1977; Ursino *et al.*, 1982). In soybean, however, Streeter (1981) has shown that total carbohydrate levels in soybean nodules are not affected by nitrate. But this does not hold true for all legume species and sugar levels do decline in *Phaseolus vulgaris* nodules when the plants are grown with nitrate present (Streeter, 1983). It is evident that legume species vary considerably in nodulation response to nitrate (Harper and Gibson, 1984b) and the mechanisms of nitrate inhibition may not be common among different legume-*Rhizobium* associations.

Another hypothesis that has received considerable support is that products of nitrate reduction are involved in mediating nitrate inhibition (Streeter, 1982a). Nitrite, the immediate product of nitrate reduction, binds to and inhibits leghemoglobin (Rigaud and Puppo, 1977) and nitrogenase (Trinchant and Rigaud, 1980) and therefore is a feasible candidate for mediating inhibition of specific nitrogenase activity (activity per unit of nodule mass). However, nitrite concentrations in soybean nodules are largely dependent on bacteroid nitrate reductase (NR) activity and plants inoculated with NR-positive or NR-negative strains of *R. japonicum* are equally sensitive to nitrate inhibition of specific nitrogenase activity (Streeter, 1985b). Similarly, NR-deficient *R. japonicum* mutants cannot circumvent nitrate inhibition of soybean nodulation (Streeter, 1982a; Streeter, 1985a). This research and analogous studies in other legume root nodule symbioses (Gibson and Pagan, 1977) have clearly shown that *Rhizobium* nitrate reduction is not a major factor mediating nitrate inhibition of nodulation and nitrogen fixation. Of course, these studies do not discount the possibility that products of host nitrate metabolism are involved in regulating the symbiosis (Streeter, 1982a).

A NR-deficient pea mutant E_1 (Feenstra and Jacobsen, 1980) has been useful in assessing the role of plant NR in nitrate inhibition. Nitrate inhibits nodulation but not nitrogen fixation in this mutant (Jacobsen, 1984). It was postulated that the nitrate concentration in the growth medium and/or plant was causing the inhibition of nodulation. Similarly, Gibson and Harper (1985) have suggested that the external concentration of nitrate rather than the rate of nitrate uptake has a major effect on the initial stages of soybean nodulation. Consistent with this theme, split root experiments have

shown that nitrate inhibition of nodule initiation is a localized effect (Hinson, 1975; Carroll and Gresshoff, 1983), whereas inhibition of nitrogenase activity appears to be systemic (Carroll and Gresshoff, 1983).

An interesting peculiarity of nitrate inhibition of soybean nodule formation under field conditions is that high *Rhizobium* numbers can partially alleviate blockage of nodulation (Herridge *et al.*, 1984). In this respect, an analogy can be drawn between nitrate inhibition of nodulation and host genotype blockage of nodulation in soybean. La Favre and Eaglesham (1984) have shown that non-nodulation in soybean mutant rj_1 can be partly circumvented by inoculation with high doses of *R. japonicum*.

1.4 Host Genetics Studies on Nodulation

Many *Rhizobium* mutants have been isolated and these mutants have led to an increased understanding of the *Rhizobium* attributes that are critical in nodulation and nitrogen fixation (Ronson *et al.*, 1981; Stacey *et al.*, 1982; Brewin, 1984; Rolfe and Shine, 1984; Stacey *et al.*, 1984). Studies on *Rhizobium* mutants have also been useful in highlighting host contributions to the symbiosis (Halverson and Stacey, 1984, 1985). As has been demonstrated in the preceding sections, the role of the host is considerable, but only a few host mutants have been identified. Several non-nodulating legume mutants have been reported in the literature (Nutman, 1981; Peterson and Barnes, 1981; Nambiar *et al.*, 1983; Kneen and La Rue, 1984). Ineffective variants have been identified in alfalfa (Vance *et al.*, 1984). In soybean, only four nodulation mutants have been reported in the literature and these naturally-

occurring variants comprise one non-nodulating mutant called rj_1 (Williams and Lynch, 1954; La Favre and Eaglesham, 1984) and three ineffectively nodulating mutants designated Rj_2, Rj_3 and Rj_4 (Caldwell, 1966; Vest, 1970; Vest and Caldwell, 1972).

Genetic approaches to overcome nitrate inhibition have been generally restricted to the *Rhizobium* symbiont (Gibson and Pagan, 1977; Streeter, 1982a; McNeil, 1982). Although these studies have demonstrated the futility in searching for *Rhizobium* strains for nitrate-tolerant symbioses, they have stressed a dominating contribution by the host genome in the nitrate inhibition phenomenon. The prospect of isolating nitrate-tolerant legume hosts is promising. As referred to in the previous section, nitrogen fixation appears to be nitrate tolerant in a nitrate reductase-deficient pea mutant (Jacobsen, 1984) and differential nodulation tolerance to nitrate has been demonstrated between species and between cultivars within a species (Harper and Gibson, 1984a,b; Carroll *et al.*, 1984; Gibson and Harper, 1985).

1.5 Aims of this Study

The major aim of this study was to investigate the role of the plant in the soybean-*R. japonicum* symbiosis. Special emphasis was given to the regulation of nodulation and nitrogen fixation by nitrate. Both host genetics and physiological studies on nodulation and nitrate metabolism in soybean were pursued. The arrangement of the thesis is as follows:

Chapter 2 contains the common materials and methods used in this investigation. Specific materials and methods are described in individual Results chapters.

Chapter 3 describes the procedures used to mutagenize soybean and the selection of promising M_2 populations for subsequent mutant selection.

Chapter 4 details experiments on wild-type soybeans that were designed to test hypotheses that have been proposed to explain nitrate inhibition of specific nitrogenase activity. Isolated bacteroids from nitrate- and dark-inhibited soybeans were evaluated for nitrogenase activity. The dark treatment was included as a comparative study, since loss of nitrogenase activity in dark-treated plants has been ascribed to carbohydrate deprivation. The effect of external nitrate on leghemoglobin, nitrate and nitrite levels in nodules was also assessed.

Chapter 5 further investigates nitrate- and dark-inhibition of nitrogenase activity in the wild-type and presents data indicating that 0_2 supply to the nodule is a major factor limiting nitrogenase activity in inhibited plants.

Chapter 6 describes the screening procedure used to isolate 15 independent *n*itrate-*t*olerant *s*ymbiotic (*nts*) mutants of soybean. The symbiotic, nitrate metabolism and growth properties of some of these lines are outlined.

Chapter 7 deals with a more detailed description of the symbiotic characteristics of *nts*382, with emphasis on its response to various nitrogen sources and to two different strains of *R. japonicum*.

Chapter 8 describes the mode of inheritance of the mutant character in four of the *nts* lines.

Chapter 9 reports on the isolation of two independent constitutive nitrate reductase mutants. The nitrate metabolism and nodulation characteristics of these mutants were assessed.

Chapter 10 describes the isolation and initial characterization of three non-nodulating mutants of soybean.

Chapter 11 integrates the significance of the findings in a brief, general discussion.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

The following chapter describes the original biological material and the common culturing methods used during this investigation. Detailed plant culture methods, mutagenesis procedures, harvest parameters, biochemical assays and data analysis are described in individual chapters.

2.1 Plant material. The parent cultivar of soybean (*Glycine max* (L.) Merrill) used throughout this study was Bragg. Seed was originally supplied by Dr. David Herridge (NSW Dept. of Agriculture, Tamworth). In Chapter 9, constitutive nitrate reductase-deficient mutant nr_1 (Nelson *et al.*, 1983) and its parent cultivar Williams were also used. Williams and nr_1 seed was supplied by Dr. D. Herridge and Dr. Sarah Ryan (CSIRO, Canberra), respectively.

2.2 Plant culture. Plants were cultured in the field, or in the glasshouse using river sand or vermiculite as a growth medium. The nutrient solution administered in glasshouse experiments was the same as that used by Herridge (1977). The composition of full-strength nutrient solution is listed in Table 2.1. During the first two weeks of plant growth in pot experiments, the nutrient solution was applied at one quarter-strength, except for $CaCl_2$ which was administered at full-strength. The pots received full-strength nutrient solution thereafter. Leonard jars were constructed as described by Gibson (1980) and contained one-third strength nutrient solution. KNO_3, NH_4Cl, NH_4NO_3, KCl (control) and urea were added to the nutrient solution as required.

Table 2.1: The composition of full-strength plant nutrient solution (Herridge, 1977).

Table 2.1:

Chemical[a]	Final concentration (mg. litre^{-1})
KH_2PO_4[b]	17.0
K_2HPO_4[b]	21.8
KCl[b]	18.7
$MgSO_4.7H_2O$[b]	123.3
$CaCl_2$[b]	27.7
ferric monosodium salt of EDTA[c]	8.7
H_3BO_3[d]	71.5×10^{-2}
$MnCl_2.4H_2O$[d]	45.3×10^{-2}
$ZnCl_2$[d]	2.8×10^{-2}
$CuCl_2.2H_2O$[d]	1.3×10^{-2}
$NaMoO_4.2H_2O$[d]	0.6×10^{-2}

[a] chemicals were prepared as stock solutions and diluted in tap water.

[b] administered from 1M stock solutions

[c] administered from a 4,000 times stock solution

[d] these chemicals were collectively prepared in a 4,000 times stock solution.

2.3 *Rhizobium japonicum* strains. Four strains were used during this study; these were CB1809 (= USDA136), USDA110, USDA201 and USDA257. Strains CB1809 and USDA110 are slow-growing isolates, whereas strains USDA201 and USDA257 are fast-growers. *R. japonicum* strain CB1809 is the inoculant strain used in the Australian soybean industry and was obtained from John Brockwell (CSIRO, Canberra). Strain USDA110 was supplied by Dr. John Streeter (Ohio State University, Wooster, USA) and strains USDA201 and USDA257 were obtained from Dr. Barry Rolfe (RSBS ANU, Canberra).

2.4 Culture of *R. japonicum* strains. Strains were generally cultured in Bergersen's Modified Medium (BMM) (Bergersen, 1961). The composition of BMM is listed in Table 2.2. Purity of cultures was verified by growth rates, colony appearance, inability to grow on LBG plates and resistance or sensitivity to various antibiotics. The constituents of LBG (Luria broth with glucose) are listed in Table 2.3 (Miller, 1972). Strain USDA110 cultures were also confirmed using phage 110Ø3. This phage was obtained from Dr. E. Appelbaum (Agrigenetics, Madison, Wisconsin, USA) and it specifically lyses *R. japonicum* strain USDA110. To test for lysis, a drop of USDA110 BMM culture was spread over a FGM (Table 2.4) plate, and then a small drop of phage titre (containing approximately 10^6 - 10^8 plaque-forming units) was dispensed onto the inoculated plate and allowed to dry. The plate was sealed and incubated at 30^oC for about 5 days. Lack of growth in the area of the plate that received phage confirmed the identity of the strain.

Table 2.2: Bergersen's Modified Medium (BMM). The pH of the medium was adjusted to 6.9.

Table 2.2:

Chemical*	Final concentration (mg/l)
$Na_2HPO_4.12H_2O$	360
$MgSO_4.7H_2O$	80
thiamine-HCl	1
biotin	0.1
$FeCl_3.6H_2O$	3
$CaCl_2.2H_2O$	40
$MnSO_4.H_2O$[a]	1
H_3BO_3[a]	0.3
$ZnSO_4.7H_2O$[a]	0.3
$NaMoO_4.2H_2O$[a]	0.025
$CuSO_4.5H_2O$[a]	0.025
$CoCl_2.6H_2O$[a]	0.025
sodium glutamate	500
mannitol	10000
yeast extract	500

*those chemicals that were indexed by the letter a were added to the medium from a collective trace elements stock solution (Gresshoff and Doy, 1974). The composition of BMM for plates was the same as in the Table except that the concentration of mannitol was 3g/l (instead of 10g/l). The agar concentration for plates was 15g/l.

[a]N-B5 stock solution adapted from Gamborg and Eveleigh (1968).

b,c,d iron chelate, vitamins and trace elements as described by Gresshoff and Doy (1974).

Table 2.3: Luria Broth with glucose (LBG). The pH of the medium was adjusted to 7.0.

Table 2.3:

Compound	Final concentration (g/l)
Peptone	10
NaCl	5 (85mM)
Yeast extract	5
Glucose	5
Agar	10

Table 2.4: Fast growth medium (FGM) for *Rhizobium*. The pH of the medium was adjusted to 6.5.

Table 2.4:

Chemical*	Final concentration (mg/l)
$NaH_2PO_4.2H_2O$ [a]	150
KI [a]	0.75
KCl [a]	500
Na_2SO_4 [a]	150
$MgSO_4.7H_2O$ [a]	250
$CaCl_2.2H_2O$ [a]	150
$FeSO_4.7H_2O$ [b]	2.79
Na_2EDTA (disodium ethylene-diamine tetra-acetate) [b]	3.73
myo-inositol [c]	100
thiamine-HCl [c]	11
nicotinic acid [c]	1
pyridoxine-HCl [c]	1
biotin	0.1
$MnSO_4.H_2O$ [d]	1
H_3BO_3 [d]	0.3
$ZnSO_4.7H_2O$ [d]	0.3
$NaMoO_4.2H_2O$ [d]	0.025
$CuSO_4.5H_2O$ [d]	0.025
$CoCl_2.6H_2O$ [d]	0.025
sodium gluconate	10.9×10^3
$(NH_4)_2SO_4$	660
MOPS (buffering agent)	10.5×10^3
yeast extract	1.0×10^3
agar	15.0×10^3

*those chemicals indexed by the same letter were added to the medium from a common stock solution.

[a] N-B5 salts adapted from Gamborg and Eveleigh (1968)

[b,c,d] iron chelate, vitamins and trace elements as described by Gresshoff and Doy (1974)

2.5 Inoculation of plants. Seeds and/or seedlings were inoculated with *R. japonicum* BMM liquid or peat cultures. Sterilized bags of dry peat (40g size) were obtained from Agricultural Laboratories Pty Ltd., Regents Park, NSW. Diluted or pure *R. japonicum* BMM liquid culture was injected into the peat (45mls per bag) and the bags were subsequently incubated in the dark at 28°C for 10 to 14 days. After incubation, the peat contained about 10^9 *R. japonicum* cells per gram and the identity of the strain was confirmed by the methods described above. Mature peat cultures were stored in the cold room (4°C) until use. Inoculant was applied to pots as a slurry (peat culture mixed with plant nutrient solution). Each pot received approximately 10^8 - 10^9 cells of *R. japonicum* at planting and again at three to five days after planting.

2.6 Chemicals used throughout this investigation were obtained from either Sigma Chemical Co. (Missouri, USA), Ajax Chemicals (Australia) or BDH (Australia). Gases were obtained from CIG (Australia).

CHAPTER THREE

MUTAGENESIS OF SOYBEAN (cv. BRAGG)

3.1 Introduction

Microbial genetics and plant physiology, biochemistry and anatomy have been used to study the regulation of nodule development and function in legumes (see Chapter 1). Host genetics also has enormous potential to contribute by fragmenting these complex phenomena into simple steps. However, this latter approach has been limited by the lack of host nodulation mutants. Only four nodulation mutants have been reported in soybean; these are all naturally-occurring variants and comprise of one non-nodulation mutant (Williams and Lynch, 1954) and three strain specific ineffectively-nodulating mutants (Caldwell, 1966; Vest, 1970; Vest and Caldwell, 1972). Undoubtedly, other variants do exist in nature, but the low frequency makes it impractical to identify and recover these mutants. Therefore, induced mutagenesis is required to decrease the population size that needs to be screened.

In this study, soybean seeds were treated with either ethyl methanesulphonate (EMS), sodium azide (NaN_3) or gamma-rays. EMS is an alkylating agent (Kamra and Brunner, 1977) that has been used effectively to mutagenize soybean (Lee and Halloran, 1975, 1977; Constantin, 1976; Ryan and Harper, 1983). Similarly, gamma-rays have been used successfully to induce mutations in soybean (Constantin, 1976; Ryan and Harper, 1983). NaN_3 is a powerful mutagen in other higher plant species (Nilan *et al.*, 1973; Kleinhofs *et al.*, 1978; Sarma *et al.*, 1979), but its effectiveness in soybean has not been well documented. Ryan and Harper (1983) treated seeds with NaN_3 and since the resulting M_1 plants were devoid of a reliable indicator of mutation (e.g. chlorophyll-deficient sectors), the M_2

generation was not assessed for mutation frequencies. However, NaN_3 is probably a highly efficient mutagen in some species because it induces base substitution mutations (Kleinhofs and Smith, 1976) without chromosome aberrations (Nilan *et al.*, 1973). In contrast, EMS and gamma-rays do cause chromosome breakages (Gaul, 1977). Indeed, Sander and Muehlbauer (1977) showed in pea that NaN_3 was as efficient as gamma-rays in inducing mutants in the M_2 generation, but produced fewer stunted and variant plants in the M_1 generation. Clearly, different mutagens inflict varying degrees of physiological damage on M_1 plants and it is plausible that NaN_3 may induce mutations in soybean without producing various M_1 anomalies that are characteristic of other mutagens.

This chapter describes the procedures used to mutagenize soybean. M_1 germination and M_2 mutation frequencies were assessed for the three different mutagens.

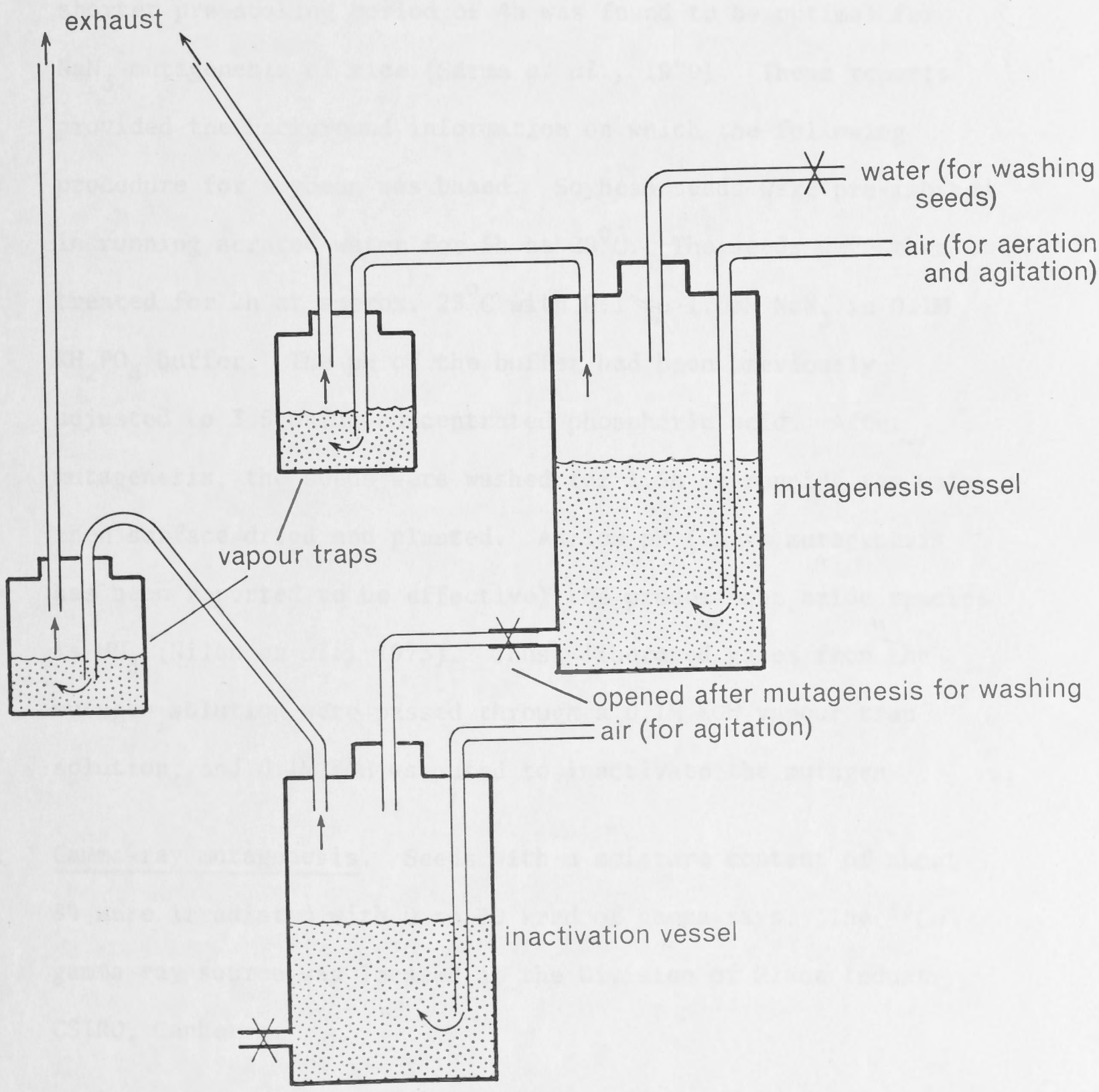
3.2 Materials and Methods

3.2.1 Plant material. Soybean cv. Bragg was used in the mutagenesis experiments described below.

3.2.2 Chemical mutagenesis. Ethyl methanesulphonate (EMS) and sodium azide (NaN_3) were used to mutagenize seeds. The general procedure involved pre-soaking in water, treatment in mutagen-containing buffer and post-washing in water. Control treatments were identical except that the mutagen was excluded from the buffer. Mutagens were handled in the fumehood and the standard apparatus used for chemical mutagenesis is illustrated in Fig. 3.1. During pre-soaking, mutagenesis and post-washing of the seeds, the solutions were vigourously aerated.

a) EMS mutagenesis. The seeds were pre-soaked in aerated running tap water for 12h at 28°C, and then treated at approx. 25°C for 4 to 5h with freshly-prepared EMS solution in 0.1M KH_2PO_4 buffer (pH = 6.0). The concentration of EMS ranged from 0.4% to 0.5%. Following mutagenesis, the seeds were washed for 4.5h in aerated running tap water and then planted wet or surface-dried. The mutagen solution was aerated during mutagenesis and the liberated gases were passed through a vapour trap (Fig. 3.1). The vapour trap solution was 10% sodium thiosulphate ($Na_2S_2O_3$) in 2% NaOH. Thiosulphate inactivates EMS (Ehrenberg and Wachtmeister, 1977) and 2% NaOH was included since this treatment prevents degradation of thiosulphate (Konzak and Narayanan, 1977). After mutagenesis, the EMS solution was inactivated by adding it to a concentrated solution of $Na_2S_2O_3$ and NaOH. The final concentrations of $Na_2S_2O_3$ and NaOH were 10% and 2%,respectively.

Figure 3.1: Fumehood apparatus for chemical mutagenesis. Pre-imbibed seeds and mutagen solution were placed in the mutagenesis vessel. The solution was aerated throughout mutagenesis, and the liberated gas was passed through a vapour trap containing inactivation solution (see text) and exhausted in the fumehood. After mutagenesis, the mutagen solution was drained away from the seeds and detoxified in the inactivation vessel. The remaining seeds were washed with tap water. Inactivated mutagen solutions were discarded down the fumehood sink with copious amounts of water.


exhaust
water (for washing seeds)
air (for aeration and agitation)
mutagenesis vessel
vapour traps
opened after mutagenesis for washing
air (for agitation)
inactivation vessel

b) NaN_3 mutagenesis. NaN_3 has been reported to be mutagenic in barley at pH 3.0 but not a pH 11.0 (Nilan *et al.*, 1973). Subsequently, Kleinhofs *et al.* (1978) successfully mutagenized pea seeds by pre-soaking for 12h prior to treatment for 2h with 1mM NaN_3 in 0.1M phosphate buffer (pH 11.0). However, a shorter pre-soaking period of 4h was found to be optimal for NaN_3-mutagenesis of rice (Sarma *et al.*, 1979). These reports provided the background information on which the following procedure for soybean was based. Soybean seeds were pre-imbibed in running aerated water for 5h at 28°C. The seeds were then treated for 2h at approx. 25°C with 0.1 to 1.0mM NaN_3 in 0.1M KH_2PO_4 buffer. The pH of the buffer had been previously adjusted to 3.5 using concentrated phosphoric acid. After mutagenesis, the seeds were washed for 1.5h in running tap water, then surface-dried and planted. At low pH (where mutagenesis has been reported to be effective) the predominant azide species is HN_3 (Nilan *et al.*, 1973). Thus, liberated gases from the mutagen solution were passed through a 0.1M KOH vapour trap solution, and 0.1M KOH was used to inactivate the mutagen.

3.2.3 Gamma-ray mutagenesis. Seeds with a moisture content of about 8% were irradiated with 0 to 30 krad of gamma-rays. The ^{60}Co gamma-ray source was located at the Division of Plant Industry, CSIRO, Canberra.

3.2.4 Plant culture and seed harvesting. Mutagenesis effects can be categorized into two classes: physiological damage and point or chromosomal mutations. Physiological damage or plant injury is generally confined to the M_1 generation, whereas mutations are transferred through to subsequent generations (Gaul, 1977).

Although mutation frequencies are critical, these cannot be properly assessed in the M_1, since soybean is diploid (Lackey, 1981) and most mutations are recessive (Gottschalk and Wolff, 1983). However, physiological damage in the M_1 generation can be quantified in a number of ways (Gaul, 1977), including germination frequencies. Therefore, in preliminary experiments designed to determine the appropriate dosage of the various mutagens, M_1 germination tests were conducted in the glasshouse. In glasshouse experiments, M_1 plants were cultured in sand or vermiculite and received 5mM KNO_3-supplemented nutrient solution (Chapter 2) three times a week, and tap water every other day. Subsequently, in large-scale experiments, M_1 seed was planted in the field either at the Breeza Experimental Station (NSW Dept. of Agriculture) or at the Botany Dept., ANU, Canberra. Germination frequencies and plant height at maturity were used as indicators for plant injury in the M_1. After the M_1 crop matured, M_2 families of seed were harvested. Each M_2 family comprised of 50 (or less) seeds that were randomly sampled from each individual M_1 plant. Not all of the seed was harvested as M_2 families and the remaining seed within each mutagenized population (i.e. M_1 plants that received the same mutagenic treatment) were mechanically harvested to give bulk M_2 populations. To assess mutation frequencies, samples of M_2 plants were cultured on N-free nutrients (Chapter 2) in sand trays (63cm long, 23cm wide, 6.5cm deep) in the glasshouse and unifoliolate leaves were examined for chlorophyll deficiency. The frequency of M_2 chlorophyll-deficient variants for each mutagenized population was expressed as a percentage.

3.3 Results and Discussion

A common effect of mutagens is to decrease M_1 survival (Gaul, 1977). Accordingly, increasing the dose of gamma-rays, NaN_3 or EMS resulted in decreased M_1 germination (Table 3.1).

Based on results of the preliminary experiments illustrated in Table 3.1, several mutagenic treatments were selected and used in large scale mutagenesis experiments. During the 1981-1982 season, M_1 seeds were planted in the field at the Breeza Experimental Station (NSW Dept. of Agriculture). To assess physiological damage in the M_1, germination frequencies were determined 62 days after planting and plant height was measured when M_2 seed was harvested. These parameters are listed in Table 3.2. Consistent with preliminary experiments (Table 3.1), increasing the dose of all three mutagens resulted in decreased germination frequencies in comparison to controls (Table 3.2). Comparing the 0 krad control (planted as dry seed) and the chemical mutagenesis controls (Table 3.2), it is evident that soaking the seeds in water and buffer prior to planting reduced plant height; perhaps physiological damage occurred or nutrients were leached from the seed during soaking. The effect of the mutagenic treatments on plant height was less marked than on germination, however, higher dose treatments produced corresponding decreases in plant height. This phenomenon was more pronounced with gamma-rays than with the chemical treatments (Table 3.2). Furthermore, there was considerable variation in the degree of M_1 leaf senescence at the time M_2 seed was harvested. Leaves of control plants and NaN_3-treated plants were fully senesced. In contrast, EMS-treated and particularly gamma-ray-treated plants were characterized by

Table 3.1: The effect of gamma-rays, sodium azide (NaN_3) and ethyl methanesulphonate (EMS) on M_1 germination in the glasshouse. M_1 seeds were mutagenized as described in Materials and Methods.

Table 3.1

Mutagen	Dose	% Germination
gamma-rays[b]	0 krad	100
	5 krad	92.8
	10 krad	85.6
	15 krad	75.3
	20 krad	55.7
	30 krad	30.9
sodium-azide[c]	buffer, 2h	100
	0.1mM NaN_3, 2h	93.2
	0.2mM NaN_3, 2h	100.8
	0.3mM NaN_3, 2h	74.5
	0.4mM NaN_3, 2h	39.0
	1.0mM NaN_3, 2h	26.0
ethyl methane-sulphonate[c]	buffer, 4h	100
	0.4% EMS, 4h	55.8

[a] 60-100 seeds were mutagenized per treatment; germination (survival) is expressed as a percentage of control

[b] assessed 7 weeks after planting and culture in 25cm diameter pots of river sand

[c] assessed 3 weeks after planting and culture in trays (63cm long, 23cm wide and 6.5cm deep) of vermiculite.

Table 3.2: The effect of gamma-rays, sodium azide (NaN_3) and ethyl methanesulphonate (EMS) on M_1 germination and plant height. M_1 seeds were planted in the field at the Breeza Experimental Station (NSW Dept. of Agriculture).

Table 3.2

Mutagen	Dose	% germination[a]	plant height[b] (cm)
gamma-rays	0 krad	100	78
	15 krad	99	55
	20 krad	92	52
	25 krad	35	41
sodium azide	buffer, 2h	100	62
	0.2mM NaN_3, 2h	78	60
	0.5mM NaN_3, 2h	43	58
	1.0mM NaN_3, 2h	18	56
ethyl methane-sulphonate	buffer, 4h	100	57
	0.44% EMS	53	54

[a] germination (survival) frequencies were estimated 62 days after planting and are expressed in the Table as a % of controls

[b] plant height (from ground-level to the uppermost node) was measured at seed harvest and the $LSD_{0.05}$ for plant height was equal to 4.

delayed leaf senescence, such that at the time of harvest some parts of the plants were still green.

An additional EMS-mutagenized crop was grown in the field at the Botany Dept., ANU, Canberra, during the 1982-1983 season. The controls for this experiment were devoured by rabbits, and therefore germination frequencies and plant height in EMS-treated and control plants could not be compared. However, the mutation frequency in progeny from this mutagenized crop was compared with the mutagenized M_2 populations derived from Breeza.

The frequency of chlorophyll-deficient variants in the M_2 was used as an indicator of mutation frequencies. EMS treatments were most effective in inducing chlorophyll-deficient variants, and the frequency was related to the dose that the M_1 seeds received (Table 3.3). M_1 dosages of 0.44% EMS for 4h and 0.5% EMS for 5h resulted in 0.9% and 2.8%, respectively, of M_2 plants being chlorophyll-deficient. Although not as spectacular as EMS, gamma-rays also produced chlorophyll-deficient variants in the M_2. M_2 population GR25 was derived from 25 krad mutagenesis and had 0.3% chlorophyll-deficient variants (Table 3.3). In a separate experiment, the estimated mutation frequencies in M_2 populations GR15 and GR20 (derived from 15 krad and 20 krad mutagenesis, respectively) was similar to that observed in M_2 population GR25 (data not shown).

Although NaN_3 is an efficient mutagen in other plant species (Nilan *et al.*, 1973; Kleinhofs *et al.*, 1978; Sarma *et al.*, 1979), this chemical was not effective in generating chlorophyll-deficient variants in soybean. Despite causing reductions in M_1 germination (Tables 3.1 and 3.2), M_2 populations

Table 3.3: Frequency of chlorophyll-deficient variants in M_2 populations derived from gamma-ray, NaN_3- or EMS-mutagenesis. Plants were cultured as described in Materials and Methods, and only primary leaves were assessed for chlorophyll-deficiency. The frequency of variants in each population is expressed as a percentage of the total number of plants screened.

Table 3.3

M_2 population[a]	M_1 seed dose	M_2 chlorophyll-deficient variants (% of total plants screened)[b]
control[c]	buffer, 2-4h	0.0
GR25	25 krad	0.3
NaN_3-1	0.2mM NaN_3, 2h	0.0
NaN_3-2	0.5mM NaN_3, 2h	0.0
NaN_3-3	1.0mM NaN_3, 2h	0.2
EMS-population 1	0.44% EMS, 4h	0.9
EMS-population 2	0.5% EMS, 5h	2.8

[a] all M_2 populations were derived from the Breeza field experiment except EMS-population 2 which was M_2 progeny of the Canberra field experiment

[b] sample size = 1080, 3000, 540, 540, 540, 540 and 2312 plants, respectively

[c] the control population comprised of plants from the EMS- and NaN_3-buffer controls.

derived from NaN_3 treatment had few or no chlorophyll-deficient variants (Table 3.3). The highest NaN_3 dose reduced M_1 germination by about 75% (Tables 3.1 and 3.2), but resulted in only 0.2% chlorophyll-deficient plants in the M_2 (Table 3.3). Azide is an inhibitor of catalase and peroxidase enzymes (Sarma *et al.*, 1979) and the decreased M_1 germination observed here may have been due to inhibition of respiration, rather than to mutagenesis. Under similar conditions, higher doses of NaN_3 were required to decrease germination in rice (Sarma *et al.*, 1979) than those described in this chapter for soybean. Perhaps respiration in soybean is more sensitive to azide and the concentration of NaN_3 required for mutagenesis greatly exceeds the level that can be tolerated by respiratory-related enzymes. In sensitive species, NaN_3 is a base-substitution mutagen, but its mutagenicity is highly specific, since some base substitutions and not others in *Salmonella typhimurium* are reverted by azide (Kleinhofs and Smith, 1976). Perhaps in NaN_3-treated soybean chlorophyll-deficiency is not a reliable monitor of mutation in general. Nevertheless, the lack of positive results indicating mutagenicity precluded the use of NaN_3-derived populations in screening programs for host nodulation mutants.

Based on the results presented in this chapter, EMS-derived M_2 populations were chosen as the plant material most likely to harbour nodulation and nitrogen metabolism mutants. The recovery of these mutant types from EMS-derived M_2 populations is described in Chapters 6, 9 and 10.

CHAPTER FOUR

NITROGENASE ACTIVITY BY BACTEROIDS ISOLATED FROM NITRATE-AND DARK-INHIBITED SOYBEAN (*GLYCINE MAX* (L.) MERR.) PLANTS.

4.1 Introduction

Although small amounts of nitrate stimulate legume-*Rhizobium* symbioses, the generality is that nitrogen fixation is inversely related to soil nitrate levels (Herridge, 1982; Carroll and Gresshoff, 1983; McNeil and La Rue, 1984). Continuous exposure to nitrate decreases nodule number and mass, as well as specific nitrogenase activity (activity per unit of nodule mass) (Ursino *et al.*, 1982; Carroll and Gresshoff, 1983). Furthermore, administering nitrate to N_2-dependent plants decreases specific nitrogenase activity (Streeter, 1981; Carroll and Gresshoff, 1983) and ultimately causes nodule senescence (Chen and Phillips, 1977). It is not clear whether nitrate inhibition of nodule development and specific nitrogenase activity are coordinately or separately regulated, however, two major hypotheses have been proposed to explain the nitrate inhibition phenomenon. These are that either products from nitrate reduction (Streeter, 1982a) or a presumptive decline in carbohydrate supply associated with nitrate assimilation mediate inhibition of the symbiosis (Gibson and Pagan, 1977).

Nitrite, the first intermediate product in the nitrate reduction pathway, is a possible candidate for regulating specific nitrogenase activity because it binds to and inhibits leghemoglobin (Rigaud and Puppo, 1977) and nitrogenase (Trinchant and Rigaud, 1980). The carbohydrate deprivation hypothesis also merits consideration since both nitrate and dinitrogen assimilation are dependent on photosynthate supply. Generally, treatments which increase photosynthesis also stimulate nitrate reduction (Nicholas *et al.*, 1976ab) and nitrogen fixation (Hardy and Havelka, 1976). Consistent with a carbon limitation model, treatment of N_2-dependent soybean plants with nitrate results in decreased translocation of ^{14}C-photosynthate

to the nodules (Small and Leonard, 1969; Latimore *et al.*, 1977; Ursino *et al.*, 1982). But this phenomenon may be due to a loss in the sink capacity of nodules rather than to a reduced supply of carbohydrate in the nodules. Indeed, Streeter (1981) showed that total carbohydrate levels in soybean nodules were not decreased by nitrate treatment. However, the effect of nitrate on nodule glucose levels was consistently negative (Streeter, 1981), and pool sizes of other carbohydrates in nodules may not be indicative of carbon flow rates to the bacteroids. Therefore, these results indicated that there is not a substantial deficit of carbohydrates in nodules of nitrate-inhibited soybeans, but they do not preclude the possibility that the bacteroids in nitrate-inhibited plants are deprived of carbohydrate.

It has been reported that isolated bacteroids from nitrate-inhibited pea plants, supplied with ATP and dithionite (as an electron donor), were fully functional in nitrogenase activity (Houwaard, 1980). These results were consistent with the carbohydrate deprivation hypothesis, since the amount of active nitrogenase enzyme was not lower in the nodules of nitrate-inhibited plants (Houwaard, 1980). On the other hand, these results are also compatible with a hypothesis that another regulatory factor(s) (other than carbohydrate supply and the amount of active nitrogenase) limits the function of bacteroids *in vivo*.

Early investigations indicated that endogenous substrates, retained in the endophyte during extraction, were capable of supporting nitrogenase activity in isolated *R. japonicum* bacteroids (Bergersen, 1974). These bacteroids were prepared in buffer containing sucrose

(and no other carbon source), but the absence of a detectable bacteroid invertase implied that endogenous substrates, and not sucrose, were supporting nitrogenase activity (Bergersen, 1974). More recently, however, experiments on the metabolism of ^{14}C-labelled photosynthate in soybean nodules showed that sucrose may be metabolized by bacteroids *in planta* (Reibach and Streeter, 1983), despite the lack of detectable invertase in isolated bacteroids (Streeter, 1982b).

The following results in this chapter using a carbon-free extraction buffer show conclusively that nitrogenase activity in isolated *R. japonicum* bacteroids can be supported by endogenous substrates. This phenomenon was used to investigate whether or not bacteroids are deprived of carbohydrate when N_2-dependent soybeans are treated with nitrate-containing nutrient solution or for extended periods of darkness. Dark-treatment represents a good comparative system for studying nitrate effects, since loss of nitrogenase activity in dark-treated plants may be due to a lowered carbohydrate status in the plant (Schweitzer and Harper, 1980).

4.2 Materials and Methods

4.2.1 Plant culture. Soybeans (*Glycine max* (L.) Merr.) were cultured in the glasshouse in 25cm diameter pots of vermiculite. Glasshouse temperatures were held between 14°C and 30°C, and incandescent bulbs supplemented natural light such that the photoperiod was 16h. The pots were inoculated with peat cultures of *Rhizobium japonicum* strain CB1809 (=USDA136) at planting and again three to five days later (Chapter 2). The pots were watered twice a week with nitrogen-free nutrients and received tap water every other day. The composition of the nutrient solution was the same as that used by Herridge (1977). All nutrients except for $CaCl_2$ were administered at one quarter strength for the first two weeks after planting and at full strength thereafter. After 6 to 8 weeks of plant growth, plants were treated for 0 to 6 days in the dark (28°C) or with 20mM KNO_3-supplemented nutrient solution. Once nitrate treatment had commenced, nitrate and control pots were watered daily with 1.4 litres of nutrient solution.

4.2.2 Enzyme assays and metabolite determinations. Nitrogen fixation was estimated using the acetylene reduction assay (Hardy *et al.*, 1968). Detached nodules or nodules attached to decapitated root systems were assayed in 6% acetylene. Leghemoglobin content of nodules was determined using the fluorometric assay described by La Rue and Child (1979), except that a xenon excitation lamp was used instead of a mercury-xenon lamp. Nitrate and nitrite contents of nodules were determined by the procedures described by Cataldo *et al.* (1975) and Manhart and Wong (1980), respectively.

4.2.3 Preparation and assaying of bacteroids. Bacteroids were prepared using the procedure described by Bergersen and Turner (1973) with some modifications. Six grams of nodules were homogenized in 40mls of extraction buffer under N_2 in a modified omni-mixer. The extraction buffer consisted of 0.15M NaCl, 50mM KH_2PO_4, 2mM $MgCl_2$, 0.5mM $CaCl_2$, 4% polyvinylpyrrolidone (PVP) and 20mM sodium dithionite ($Na_2S_2O_4$) with pH adjusted to 7.3. A preliminary experiment verified that the osmotic potential of the extraction buffer approximated that of macerated soybean nodules (data not shown). The nodule homogenate was filtered through eight layers of cheese-cloth and then centrifuged at approximately 10g for 5mins to remove large particles. The supernatant was discarded and the bacteroids were re-suspended in washing buffer. The washing buffer was the same as the extraction buffer, except that PVP and $Na_2S_2O_4$ were omitted. The bacteroid preparation was centrifuged again at 2,000g for 5mins and the resulting pellet was resuspended in washing buffer, such that there was 1mg of bacteroid protein per ml in the final preparation. Protein was determined by the method of Lowry *et al.* (1951). Bacteroids (approximately 0.5mg of protein) were assayed in 1ml of washing buffer containing the required carbon and/or nitrogen sources. The carbon sources used were sucrose, D-glucose, sodium succinate, L-arabinose, pyruvic acid and sodium gluconate, and KNO_3 and NH_4Cl constituted the nitrogen sources. Stocks of these compounds were made up in washing buffer and adjusted to pH7.3, prior to the nitrogenase assay. The assay chamber consisted of 25ml glass scintillation vials stoppered with rubber Supa-seals. The gas atmosphere in the vials was 5% C_2H_2 and 0.2% to 1.5% O_2 in N_2. Bacteroids were generally assayed at four O_2 levels with duplicate or triplicate replication.

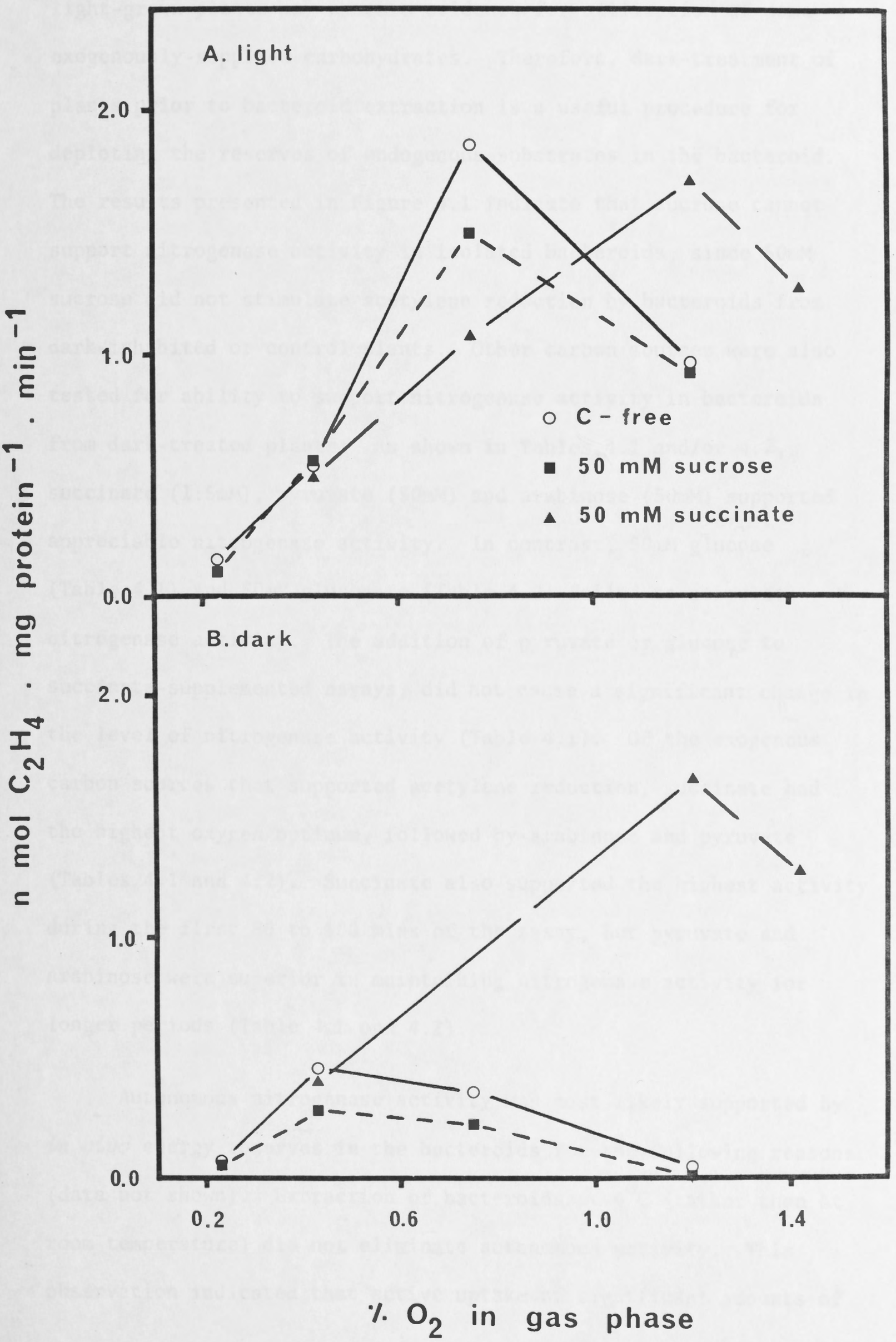
4.2.4 Statistics. Data was analyzed by analysis of variance using the statistical program Genstat (Alvey *et al.*, 1977). In one-way analyses, the least significant difference (LSD) was only computed when the F-statistic was significant at the 0.05 level.

4.3 Results

Bacteroids extracted from N_2-dependent plants and assayed without an exogenous carbohydrate had considerable nitrogenase activity (Fig. 4.1A). Clearly, endogenous substrates can support nitrogenase activity in isolated *R. japonicum* bacteroids. High autonomous nitrogenase activity was observed for at least 300mins after adding acetylene (data not shown), indicating that there were substantial energy reserves in the bacteroids. For the experiment illustrated in Fig. 4.1A, the addition of 50mM succinate to the assay buffer did not improve the maximum rate of nitrogenase activity, but the optimum O_2 level in the gas phase was higher with succinate present. Sucrose (50mM) did not increase the maximum rate of nitrogenase nor change the O_2 optimum in comparison to the carbon-free control (Fig. 4.1A).

Treatment of N_2-dependent plants for 4 days in the dark caused a 93% inhibition of specific nitrogenase activity (activity per unit of nodule mass). The mean specific nitrogenase activity of nodules on two control plants was 353nmol C_2H_4.g nodule fresh weight^{-1}. min^{-1}, compared to 24 for nodules on dark-inhibited plants. This treatment also caused a 75% inhibition of autonomous nitrogenase activity in isolated bacteroids (compare panels A and B in Fig. 4.1). When succinate was added to the assay buffer, nitrogenase activity was the same in bacteroids from dark-treated and control plants. These results confirmed that succinate can support nitrogenase activity in isolated bacteroids and indicated that the loss of autonomous nitrogenase activity in bacteroids from dark-treated plants was due to a depletion of endogenous substrates, rather than to a decrease in the amount of active nitrogenase enzyme.

Figure 4.1: The effect of succinate (50mM) and sucrose (50mM) on nitrogenase activity by isolated bacteroids. Bacteroids were extracted from plants grown under a normal photoperiod (A) and from plants that had been treated for 4 days in the dark prior to harvest. Each point in the Figure represents the mean of three replicates. The specific nitrogenase activity of nodules attached to decapitated root systems of control plants was 353 nmol C_2H_4 .g nodule fresh weight^{-1}. min^{-1}, compared to 24 for nodules of dark-inhibited plants.


A. light
B. dark
2.0
1.0
0.0
2.0
1.0
0.0
0.2
0.6
1.0
1.4
C - free
50 mM sucrose
50 mM succinate
n mol C_2H_4 . mg protein^{-1} . min^{-1}
% O_2 in gas phase

The presence of endogenous substrates in bacteroids from light-grown plants may obscure evidence for utilization of some exogenously-supplied carbohydrates. Therefore, dark-treatment of plants prior to bacteroid extraction is a useful procedure for depleting the reserves of endogenous substrates in the bacteroid. The results presented in Figure 4.1 indicate that sucrose cannot support nitrogenase activity in isolated bacteroids, since 50mM sucrose did not stimulate acetylene reduction by bacteroids from dark-inhibited or control plants. Other carbon sources were also tested for ability to support nitrogenase activity in bacteroids from dark-treated plants. As shown in Tables 4.1 and/or 4.2, succinate (1.5mM), pyruvate (50mM) and arabinose (50mM) supported appreciable nitrogenase activity. In contrast, 50mM glucose (Table 4.1) and 50mM gluconate (Table 4.2) failed to stimulate nitrogenase activity. The addition of pyruvate or glucose to succinate-supplemented assays, did not cause a significant change in the level of nitrogenase activity (Table 4.1). Of the exogenous carbon sources that supported acetylene reduction, succinate had the highest oxygen optimum, followed by arabinose and pyruvate (Tables 4.1 and 4.2). Succinate also supported the highest activity during the first 80 to 100 mins of the assay, but pyruvate and arabinose were superior in maintaining nitrogenase activity for longer periods (Table 4.1 and 4.2).

Autonomous nitrogenase activity was most likely supported by *in vivo* energy reserves in the bacteroids for the following reasons (data not shown). Extraction of bacteroids at $4^{o}C$ (rather than at room temperature) did not eliminate autonomous activity. This observation indicated that active uptake of significant amounts of

Table 4.1: The capacity of succinate, pyruvate and glucose to support nitrogenase (acetylene reduction) activity in bacteroids from plants pre-treated for 2½ days in the dark. Bacteroids were assayed at four O_2 levels, however, only the activity for the optimum O_2 concentration is listed in the Table. Each entry in the Table is the mean of two replicate assays.

Table 4.1:

Carbon Source	Optimum oxygen level (% O_2 in gas phase)	nmol C_2H_4 produced . mg bacteroid protein^{-1}.min^{-1}.	
		0 to 100mins[a]	100 to 420mins[b]
C-free	0.23	1.21 (1.10)[c]	0.07 (0.27)[c]
1.5mM succinate	0.80	5.84 (2.42)	0.50 (0.71)
50mM pyruvate	0.40	3.42 (1.84)	2.06 (1.43)
50mM glucose[d]	0.23	1.17 (1.08)	0.51 (0.70)
1.5mM succinate + 50mM pyruvate	0.80	5.47 (2.34)	0.37 (0.61)
1.5mM succinate + 50mM glucose	0.80	4.90 (2.21)	0.70 (0.82)
		($LSD_{0.05}$ = 0.25)	($LSD_{0.05}$ = 0.30)

[a] rate of nitrogenase activity from 0 to 100mins after adding acetylene

[b] rate from 100 to 420mins after adding acetylene

[c] raw data for each assay period required square-root transformation to satisfy assumptions for an analysis of variance. Means and LSD for transformed data are shown in parentheses

[d] glucose was also tested on bacteroids isolated from control plants and it failed to stimulate nitrogenase activity.

Table 4.2: The capacity of gluconate, arabinose or succinate to support acetylene reduction activity in bacteroids from plants that had been incubated in the dark for 2½ days prior to bacteroid extraction. Nitrogenase activity for the optimum (from four tested) O_2 concentrations is listed in the Table. Each entry in the Table is the mean of two or three replicate assays.

Table 4.2:

Carbon Source	Optimum oxygen level (% O_2 in gas phase)[2]	nmol C_2H_4 produced. g bacteroid protein.$^{-1}$.min^{-1}.	
		0 to 80mins[a]	80 to 250mins[b]
C-free	0.26	0.24 (-1.45)[c]	0.05 (0.21)[d]
50mM gluconate	0.22	0.11 (-2.25)	0.05 (0.22)
50mM arabinose	0.42	2.09 (0.74)	1.93 (1.39)
1.5mM succinate	0.98	2.36 (0.85)	0.24 (0.49)
		($LSD_{0.05}$ = 0.27)	($LSD_{0.05}$ = 0.14)

[a] rate from 0 to 80mins after adding acetylene

[b] rate from 80 to 250mins after adding acetylene

[c,d] raw data required $\log_e$ (c) and square-root (d) transformation to satisfy assumptions for an analysis of variance. Means and LSD for transformed data are shown in parentheses.

carbohydrates from the nodule homogenate did not occur during bacteroid extraction. Supporting this conclusion, varying the time between nodule homogenization and separation of the bacteroids did not influence autonomous nitrogenase activity. Furthermore, incubating carbohydrate-deprived bacteroids from dark-treated plants in nodule homogenate supernatant or in succinate-containing buffer did not restore autonomous nitrogenase activity.

The unequivocal demonstration of autonomous nitrogenase activity in bacteroids represents an avenue for testing whether the endophyte in nitrate-inhibited plants is deprived of carbohydrate. In subsequent experiments, investigating nitrogenase activity by bacteroids from nitrate-inhibited plants, bacteroids were assayed with and without succinate. Such an approach made it possible to distinguish between deprivation of endogenous substrate reserves and impairment in the nitrogenase apparatus (or in the succinate-utilization system of the bacteroid). Succinate was chosen as the carbon source since succinate uptake-deficient mutants of *Rhizobium* are ineffective in fixing N_2 *in planta*, implying that utilization of succinate (or another C_4-dicarboxylate such as malate) by the bacteroid is required for a functional symbiosis in at least some legume species (Ronson *et al.*, 1981; Glenn and Brewin, 1981).

As shown in Table 4.3, administering 20mM KNO_3-supplemented nutrient solution to N_2-dependent plants for 2 to 6 days caused a significant inhibition of nitrogenase (acetylene reduction) activity by intact nodules. Two days of watering with nitrate inhibited specific nitrogenase activity by 72%; 4 and 6 days on nitrate elevated the extent of inhibition to 86% and 92%, respectively (Table 4.3).

Table 4.3: The effect of nitrate treatment on nitrogenase (acetylene reduction) activity of detached nodules and isolated bacteroids. Plants were watered with 20mM KNO_3-supplemented nutrient solution for 0 to 6 days prior to harvest. Bacteroids were assayed without a carbon source (C-free) or with 1.5mM succinate, and although activity was measured at four O_2 concentrations, only the activity for the optimum O_2 level of each treatment is listed in the Table.

Table 4.3:

Days on 20mM KNO_3	Nitrogenase activity		
	detached nodules[a]	isolated bacteroids[b]	
		C-free	+ 1.5mM succinate
0	47.7 (100)[c]	1.52 (100)	7.13 (100)
2	13.5 (28)	3.28 (216)	5.66 (79)
4	6.6 (14)	1.00 (66)	2.45 (34)
6	4.0 (8)	0.09 (6)	0.62 (9)

[a] activity for detached nodules is expressed in nmol C_2H_4 produced .g nodule fresh weight^{-1}. min^{-1}. Each entry in the Table is the mean of two assays

[b] ethylene was measured approximately 100mins after adding acetylene and data are expressed as nmol C_2H_4 produced.mg bacteroid protein^{-1} .min^{-1}. Each entry in the Table is the mean of two replicate assays

[c] data in brackets are expressed as a percentage of the control (0 days on 20mM KNO_3).

In contrast, autonomous nitrogenase activity by isolated bacteroids from plants treated for 2 days on nitrate was not depressed, and was actually higher than the control in this experiment. However, after the plants had received nitrate for 4 to 6 days, the bacteroids extracted therefrom had both reduced autonomous and succinate-supported nitrogenase activity (Table 4.3). Therefore, in these later stages of nitrate-induced nodule senescence, the amount of active nitrogenase enzyme and/or the capability of succinate to support isolated bacteroid nitrogenase activity was diminished. Comparing these results (Table 4.3) to those presented in Figure 4.1, there is a distinction between the later stages of nitrate- and dark-induced nodule senescence, in that succinate was capable of restoring nitrogenase activity to bacteroids from dark-treated plants.

The activity of detached nodules for the experiment presented in Table 4.3 was lower than usual. In a repeat experiment for the 2-day nitrate treatment, the nodules were not detached and were assayed on decapitated root systems. Treatment with 20mM KNO_3 for 2 days caused a 51% inhibition of measurable specific nitrogenase activity (Table 4.4). In contrast, autonomous nitrogenase activity was decreased by only 31% and succinate-supported activity was higher in the treated than in the control. The discrepancy in the bacteroid nitrogenase activity presented in Tables 4.3 and 4.4 illustrates a limitation of the bacteroid assay system described here. Although bacteroids were assayed at four O_2 concentrations and the optimum O_2 level fell within the range tested, the true O_2 optimum in individual experiments may have been missed. Nevertheless, collectively the results indicate that the extent of inhibition of specific nitrogenase activity by the 2-day nitrate treatment could not be explained by bacteroid carbohydrate deprivation.

Table 4.4: The effect of nitrate treatment on nitrogenase (acetylene reduction) activity of isolated bacteroids and attached nodules. Treated plants were watered with 20mM KNO_3-supplemented nutrient solution for two days prior to harvest, whereas control plants received N-free nutrients. Isolated bacteroids were assayed without a carbon source (C-free) or with 1.5mM succinate. Although bacteroids were assayed at four O_2 concentrations, only the activity for the optimum O_2 level of each treatment is listed in the Table.

Table 4.4

Days on 20mM KNO_3	Nitrogenase activity		
	attached nodules[a]	isolated bacteroids[b]	
		C-free	+ 1.5mM succinate
0	431 (100)[c]	2.70 (100)	3.45 (100)
2	210 (49)	1.85 (69)	6.24 (181)

[a] whole root systems were assayed and data are expressed in nmol C_2H_4 .g nodule fresh weight^{-1} .min^{-1}. Each entry in the Table is the mean of 4 to 6 plants

[b] ethylene was measured 100mins after adding acetylene and data are expressed as nmol C_2H_4 produced .mg bacteroid protein^{-1} .min^{-1}. Each entry in the Table is the mean of two assays

[c] data in brackets are expressed as a percentage of the control.

This conclusion has since been confirmed by Schuller *et al.* (1985).

Leghemoglobin, nitrate and nitrite content of nodules for the experiment presented in Table 4.3 were also determined. A fluorometric assay for leghemoglobin was used and this involved removal of the iron from the protein to form a characteristic protoporphyrin (La Rue and Child, 1979). Therefore, the assay did not distinguish between the ferrous and ferric species of leghemoglobin; only the ferrous form is capable of oxygenation (Appleby, 1984). With this limitation in mind, a significant decrease in measurable leghemoglobin content of nodules was not evident until after 6 days of plant culture on nitrate (Fig. 4.2). Nitrate and nitrite levels in the nodules were high after 2 days of nitrate treatment, and these levels were generally maintained throughout the duration of the experiment (Fig. 4.2). During the nitrate treatment period, nitrate levels were at least ten times higher than those of nitrite (Fig. 4.2).

To determine whether the nitrate levels measured in nodules were high enough to be detrimental to bacteroid nitrogenase activity, bacteroids isolated from N_2-dependent plants were assayed in the presence of 10mM KNO_3 (Figs. 4.3 and 4.4). A 10mM NH_4Cl treatment was also included in these experiments. Figure 4.3 illustrates the effect of nitrate or ammonium on bacteroid nitrogenase activity measured in the absence of an exogenous carbon source. During the first 100mins of the assay neither nitrogen source had a significant effect (Fig. 4.3A), whereas in the subsequent 200mins nitrate, but not ammonium, caused a significant inhibition of nitrogenase activity (Fig. 4.3B). With succinate present, there was significant inhibition of nitrogenase activity by nitrate, both in the first (Fig. 4.4A) and particularly in the second (Fig. 4.4B) phase of the assay. In contrast to the carbon-free assay (Fig. 4.3), 10mM NH_4Cl

Figure 4.2: The effect of nitrate treatment on leghemoglobin (A), nitrate and nitrite (B) content of nodules. N_2-dependent plants were cultured for 0 to 6 days on 20mM KNO_3 prior to harvest. Respective LSD values (0.05 level of significance) are illustrated in the Figure.

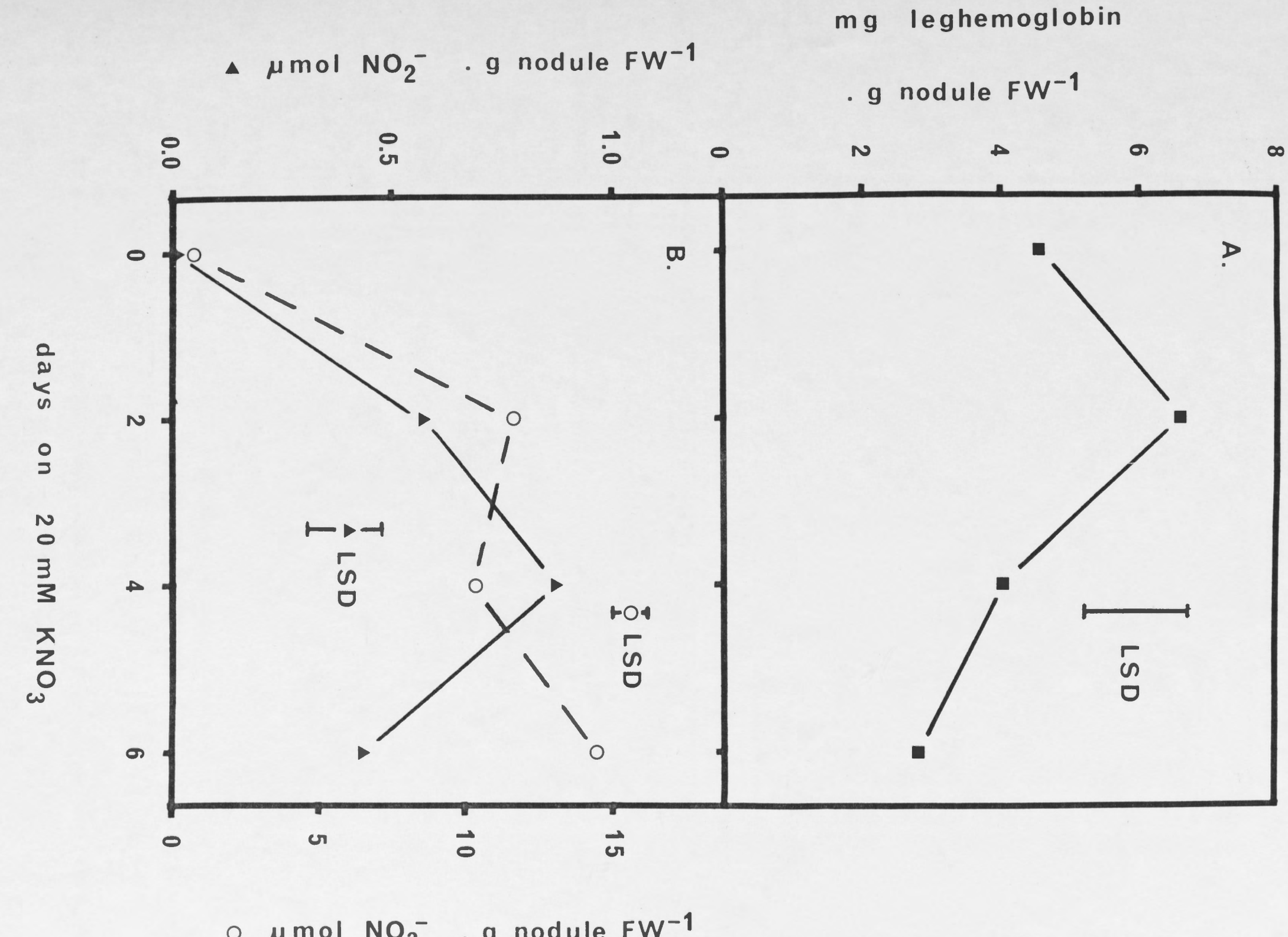

mg leghemoglobin
. g nodule FW^{-1}
A.
LSD
▲ µmol NO_2^- . g nodule FW^{-1}
B.
LSD
LSD
days on 20 mM KNO_3
○ µmol NO_3^- . g nodule FW^{-1}

Figure 4.3: The effect of KNO_3 and NH_4Cl on autonomous nitrogenase activity by isolated bacteroids. Two way analysis of variance indicated that during the first 110mins of the assay (A) there was not a significant effect (0.05% level) of nitrogen source. However, subsequently (B) there was significant nitrate inhibition. There was a significant 0_2 effect throughout the experiment, however there was no interaction between 0_2 concentration in the gas phase and the nitrogen source.

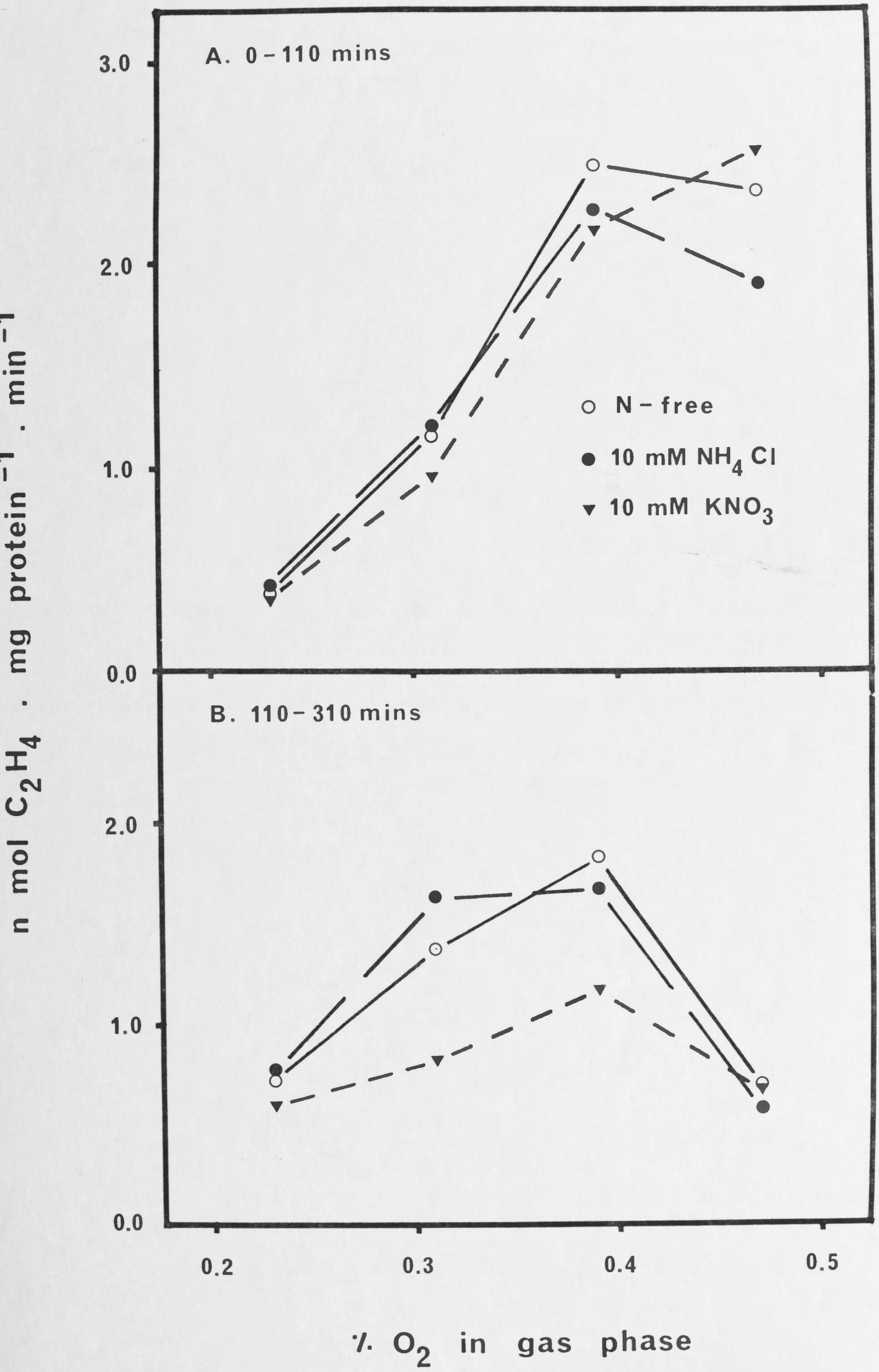
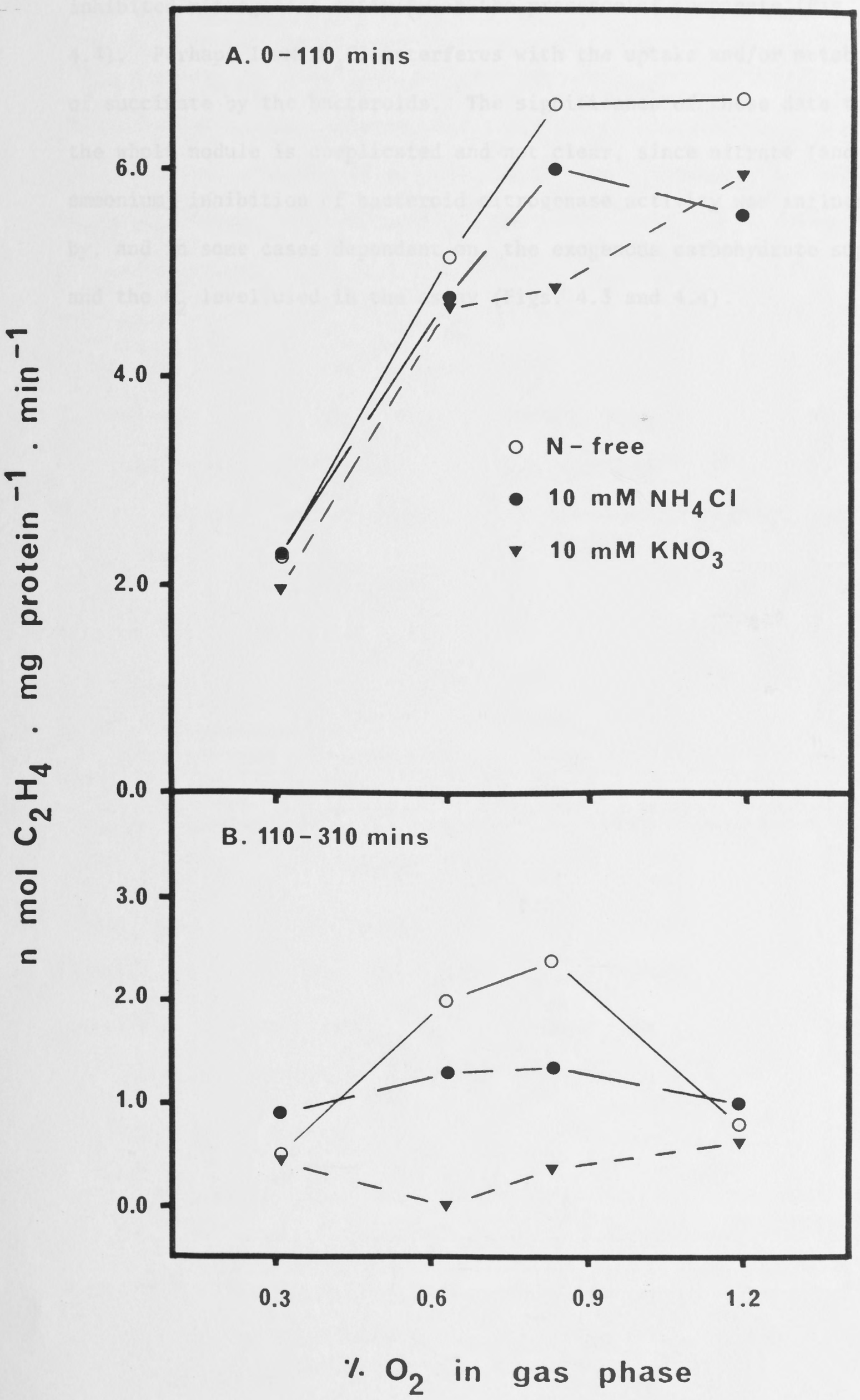

A. 0 – 110 mins
B. 110 – 310 mins
3.0
2.0
1.0
0.0
2.0
1.0
0.0
N – free
10 mM NH4 Cl
10 mM KNO3
0.2
0.3
0.4
0.5
n mol C2H4 . mg protein-1 . min-1
% O2 in gas phase

Figure 4.4: The effect of KNO_3 and NH_4Cl on succinate-supported nitrogenase activity by isolated bacteroids. During the initial 100mins of the assay (A), there was a significant effect (0.05% level) of combined nitrogen and 0_2 level in the gas phase. Subsequently (B), combined nitrogen, 0_2 concentration and interaction effects were significant.


A. 0 – 110 mins
B. 110 – 310 mins
n mol C_2H_4 . mg protein^{-1} . min^{-1}
6.0
4.0
2.0
0.0
3.0
2.0
1.0
0.0
N – free
10 mM NH_4Cl
10 mM KNO_3
0.3
0.6
0.9
1.2
% O_2 in gas phase

inhibited nitrogenase activity in the presence of succinate (Fig. 4.4). Perhaps 10mM NH_4Cl interferes with the uptake and/or metabolism of succinate by the bacteroids. The significance of these data to the whole nodule is complicated and not clear, since nitrate (and ammonium) inhibition of bacteroid nitrogenase activity was influenced by, and in some cases dependent on, the exogenous carbohydrate supply and the O_2 level used in the assay (Figs. 4.3 and 4.4).

4.4 Discussion

Clearly, endogenous energy reserves are capable of supporting considerable rates of autonomous nitrogenase activity by isolated *R. japonicum* bacteroids (Fig. 4.1). This form of activity could be largely eliminated by incubating the plants in the dark prior to bacteroid extraction (Fig. 4.1). However, succinate, pyruvate and arabinose were capable of restoring nitrogenase activity to bacteroids from dark inhibited plants (Fig. 4.1, Tables 4.1 and 4.2). Therefore, in bacteroids from plants that were treated for up to 4 days in the dark, the amount of active nitrogenase enzyme was not decreased and loss of autonomous activity was due to carbohydrate deprivation.

Although succinate uptake-deficient bacteroids of *R. trifolii* (Ronson *et al.*, 1981) and *R. leguminosarum* (Glenn and Brewin, 1981) are ineffective, other carbon sources besides C_4-dicarboxylates are capable of supporting activity by *R. japonicum* bacteroids (Tables 4.1 and 4.2). On the other hand, sucrose, glucose and gluconate failed to stimulate bacteroid nitrogenase activity (Fig. 4.1, Tables 4.1 and 4.2). Previously, it has been proposed that sucrose and glucose are capable of supporting nitrogenase activity in isolated *R. japonicum* bacteroids (Trinchant *et al.*, 1981). However, these reported experiments lacked a carbon-free control and it is likely that the activity measured was due to the presence of endogenous energy reserves.

Recent studies on the metabolism of ^{14}C-photosynthate in soybean nodules have strongly implied that sucrose and glucose are metabolized by bacteroids *in planta* (Reibach and Streeter, 1983). The results presented in this chapter indicate that sucrose and glucose cannot support nitrogenase activity in isolated *R. japonicum* bacteroids.

Perhaps sucrose metabolism by bacteroids *in planta* is linked to the formation of trehalose (Reibach and Streeter, 1983) rather than directly to nitrogenase activity. Alternatively, the ability to utilize sucrose and glucose for nitrogenase activity was lost during bacteroid isolation.

Bacteroids isolated from 2-day nitrate-inhibited plants had considerable autonomous nitrogenase activity, despite marked inhibition of nitrogenase activity by intact nodules (Tables 4.3 and 4.4). Thus, the early decline in specific nitrogenase activity in nitrate-inhibited soybeans could not be attributed to bacteroid carbohydrate deprivation. This is in contrast to the results reported by Trinchant and Rigaud (1984) for bacteroids isolated from *Phaseolus vulgaris* plants that had been treated for 24h with nitrate-containing nutrient solution. Therefore, it is important to note that the mechanism(s) of nitrate inhibition of specific nitrogenase activity may not be common between different legume species. Indeed, it has been shown that nitrate depresses the total concentrations of sugars in nodules of *P. vulgaris* plants (Streeter, 1983) but not in nodules of soybean plants (Streeter, 1981). Perhaps bacteroids in nitrate-inhibited *P. vulgaris* plants are deprived of carbohydrates. Also, the decline in the capacity of bacteroid function may be accelerated in *P. vulgaris*, as compared to *G. max*, during nitrate-induced nodule senescence.

Longer periods of soybean culture on nitrate resulted in both decreased autonomous and succinate-supported nitrogenase activity by isolated *R. japonicum* bacteroids (Table 4.3), indicating that damage did eventually occur to the microsymbiont. Thus, even in the later stages of nitrate-induced nodule senescence there was no detectable evidence of bacteroid carbohydrate deprivation, since impairment of

isolated bacteroid nitrogenase activity could not be recovered with succinate supplied in the assay buffer. These results contrasted with those for dark-treated plants in that damage (mediated by a factor other than carbohydrate supply) eventually occurred to the bacteroids after 4 to 6 days of plant culture on nitrate.

The suppression of specific nitrogenase activity by culture on nitrate was inversely related to the levels of nitrate and nitrite in the nodules (Table 4.3, Fig. 4.2B). Inclusion of nitrate in the bacteroid assay buffer eventually caused inhibition of nitrogenase activity, although the extent of inhibition was dependent upon the carbohydrate source and the 0_2 supply (Tables 4.3 and 4.4). *R. japonicum* strain CB1809 bacteroids possess nitrate reductase (Rigaud *et al.*, 1973) and the eventual inhibition was probably due to an accumulation of nitrite, which has been shown to bind reversibly to and inhibit nitrogenase (Trinchant and Rigaud, 1980). This idea is supported by the report that nitrate doesn't inhibit nitrogenase activity by isolated *R. japonicum* bacteroids that are nitrate reductase (NR)-deficient (Stephens and Neyra, 1983). Nitrite content of soybean nodules is largely dependent on bacteroid NR activity (Streeter, 1985ab). However, nitrite produced by *R. japonicum* bacteroids is not responsible for inhibition of nitrogenase activity *in planta*, since plants containing NR^+ and NR^- rhizobia are equally inhibited by nitrate (Streeter, 1985ab; Gibson, Carroll and Gresshoff, unpublished data). But, these findings do not rule out the possibility that relatively small amounts of plant-produced nitrite, or products derived thereof, effect nitrate inhibition of nitrogenase activity, perhaps by interacting with leghemoglobin. Nitrite converts ferrous leghemoglobin to the inactive ferric form

(Rigaud and Puppo, 1977), and nitric oxide may also inhibit oxygenation of leghemoglobin (Maskall *et al.*, 1977). The assay employed in this study did not detect significant decreases in leghemoglobin protein until after 6 days of plant culture on nitrate (Fig. 4.2). However, this assay was not an indicator of the biological activity of leghemoglobin *in vivo*. Thus, oxygenation of leghemoglobin may have been hampered in the early stages of nitrate-induced nodule senescence.

In the subsequent chapter, results are presented that indicate that oxygen supply limits specific nitrogenase activity in nitrate- and dark-inhibited soybean plants.

CHAPTER FIVE

OXYGEN SUPPLY TO THE NODULE LIMITS NITROGENASE ACTIVITY IN NITRATE- AND DARK-INHIBITED SOYBEANS

5.1 Introduction

Nitrogen-fixing *Rhizobium* bacteroids require O_2 for oxidative phosphorylation (Bergersen, 1974). On the other hand, the nitrogenase enzyme is O_2-labile (Bergersen, 1974) and therefore O_2 must be supplied to the bacteroids at a concentration that permits respiration without damaging nitrogenase. This requirement is satisfied by the existence of an O_2 diffusion barrier within the nodule cortex, exterior to the bacteroids (Tjepkema and Yocum, 1974; Sinclair and Goudriaan, 1981), and by the presence of leghemoglobin in the bacteroid-containing region of the nodule (Appleby, 1974). In essence, the role of leghemoglobin is to stabilize the low concentration of free O_2 around the bacteroids (Appleby, 1984).

There is evidence that the oxygen delivery system in legume nodules is not rigid and that it is subject to regulation. For example, it has been postulated that soybean nodules can adapt to changes in rhizosphere pO_2 by an unknown mechanism, such that the pO_2 inside the nodule is optimal for nitrogen fixation (Criswell *et al.*, 1976, 1977). These workers showed that continuous exposure of symbiotic soybean plants to a rhizosphere pO_2 of 0.06 atm initially decreased acetylene reduction activity by about 40%, but activity recovered to that of controls exposed to a pO_2 of 0.21 atm within 4 to 24h (Criswell *et al.*, 1976). Evidence for relatively rapid adjustment of oxygen supply into nodules has been shown to occur in some legume-*Rhizobium* symbioses (Witty *et al.*, 1984). Minchin *et al.* (1983) showed that acetylene rapidly induced declines in nitrogenase activity and root respiration in several, but not all, legume-*Rhizobium* associations. This

negative response occurred just a few minutes after adding acetylene (Minchin *et al.*, 1983) and was attributed to an increased resistance to oxygen diffusion into the nodules (Witty *et al.*, 1984). Further evidence for rapid changes in the resistance to oxygen diffusion in pea nodules was also demonstrated by gradually increasing the concentration of O_2 around the roots (Witty *et al.*, 1984).

Several environmental factors have been shown to inhibit specific nitrogenase activity in soybean. These include soil nitrate (Streeter, 1981; Chapter 4), prolonged darkness (Schweitzer and Harper, 1980; Chapter 4) and water stress (Pankhurst and Sprent, 1975a). The inhibitory effects of nitrate on specific nitrogenase activity have been ascribed to carbohydrate deprivation (Gibson and Pagan, 1977) and to inhibitory products of nitrate reduction (Streeter, 1982a). However, total carbohydrate levels in soybean nodules are not significantly affected by nitrate treatment (Streeter, 1981) and there is no evidence for depletion of energy reserves in bacteroids isolated from nitrate-inhibited soybean plants (Chapter 4). Similarly, an inhibitory product of nodule nitrate reduction has not been unequivocally shown to mediate nitrate inhibition in soybean (Streeter, 1985a, b). In contrast to nitrate treatment, there is evidence for bacteroid carbohydrate deprivation in the later stages of dark-induced nodule senescence (Chapter 4), even though nodule sugar and starch concentrations have been reported to be unaffected by incubation of the plants in the dark (Schweitzer and Harper, 1980). The data presented in this chapter indicate that oxygen supply to the nodule is a major factor limiting nitrogenase activity in nitrate- and dark-inhibited soybean plants.

5.2 Materials and Methods

5.2.1 Nitrate- and dark-induced nodule senescence. Seeds of soybean (*Glycine max* (L.) Merr.) cv Bragg were planted in 25cm diameter pots of vermiculite (12 seeds per pot). The plants were cultured in the glasshouse and temperatures were held between 14°C and 30°C and incandescent bulbs extended the photoperiod to 16h. The pots were inoculated with peat cultures of *Rhizobium japonicum* strain CB1809 (= USDA136) at planting and again 3 to 5 days later (Chapter 2). The pots were watered with N-free nutrient solution and received tap water every other day. The nutrient solution was the same as that used by Herridge (1977) and all nutrients except for $CaCl_2$ were administered at one quarter strength for the first two weeks after planting and at full strength thereafter (Chapter 2). After approximately 6 weeks of growth, nitrate and dark treatments commenced. In nitrate experiments, the pots were watered twice daily with 1.5l of N-free solution supplemented with 20mM KNO_3 or KCl for 2 days. For dark treatments, the pots were placed in the dark at 28°C for 1 or 2 days.

5.2.2 Continuous culture on nitrate. Seeds of soybean cv. Bragg were planted in 25cm diameter pots of river sand (12 seeds per pot). The pots were inoculated with peat cultures of *R. japonicum* strain CB1809 at planting and again at 4 days after planting. The pots were watered with nutrient solution (see above) three times a week for the first 3.5 weeks and then daily as the demand for water increased with plant size. KNO_3 was added to the N-free nutrient solution as required. The nitrate concentration in

the nutrient solution was maintained throughout the experiment, except that half of the pots in the 6mM KNO_3 treatment received N-free nutrients for 3 days prior to harvest. To ensure removal of residual nutrients, the pots were flushed with 1.4 litres of nutrient solution at each watering. Nitrate-treated plants were harvested 5 weeks after planting, whereas N_2-dependent plants were harvested at either 5 or 7 weeks after planting. The older N_2-dependent plants were sown 2 weeks earlier than the rest of the plants, and all plants were harvested on the same day. The plants were grown outside at a latitude of $37^{o}17'S$ with mean minima and maxima temperatures of $11.7^{o}C$ and $25.0^{o}C$. At harvest, the plants were measured for nodule number, nodule fresh weight, acetylene reduction activity and shoot and root fresh weight.

5.2.3 Acetylene reduction assay. Nitrogenase activity was estimated using the acetylene reduction assay (Hardy *et al.*, 1968). Decapitated root systems from 3 plants were pooled and placed in a 1040ml air-tight jar that contained moistened paper towel to prevent dessication of the nodules during the assay. Oxygen tensions in the jar were adjusted by flushing with industrial grade 0_2. The jars were incubated at $27^{o}C$ for 20min, prior to the addition of 6% acetylene to the atmosphere. Ethylene production was monitored 0-40min after adding acetylene. Rates of acetylene reduction appeared to be linear for the duration of the assay. The use of the acetylene reduction assay to study nitrate effects on this symbiosis has been validated by Schuller *et al.* (1985) using ^{15}N.

5.3 Results

5.3.1 Nitrate-induced nodule senescence. Treatment of 6 week-old N_2-dependent soybean plants for 2 days with 20mM KNO_3 caused a 40% inhibition in specific nitrogenase activity (activity per unit of nodule mass) relative to the 20mM KCl control plants when the decapitated root systems were assayed in 20.5% O_2 (Fig. 5.1). Increasing the atmospheric O_2 concentration during the assay recovered specific activity of nitrate-inhibited plants to a level comparable to the KCl controls (Fig 5.1). Regardless of treatment, optimum activity was observed at 32% O_2 for the range of O_2 concentrations tested. However, the stimulation in the KCl-treated plants was only marginal (Fig. 5.1) and was not significant at the 0.05 level. In contrast, increasing the assay O_2 concentration from 20.5% O_2 to 32% O_2 resulted in a significant 75% enhancement of specific nitrogenase activity in nitrate-inhibited plants. Clearly, oxygen supply was a major factor limiting specific nitrogenase activity in the early stages of nitrate-induced nodule senescence.

5.3.2 Dark-induced nodule senescence. Pretreatment in the dark for 1 or 2 days decreased specific nitrogenase activity measurable in 20.5% O_2 (Fig. 5.2). Specific nitrogenase activity in control and treated plants responded differently to supraambient O_2 concentrations (Fig. 5.2). For the range of O_2 tensions tested, optimum activity for control plants was measured in 43% O_2 (Fig. 5.2), but this activity was not significantly (0.05 level) higher than that measured in air. Optimum activity was also observed at 43% O_2 for plants pre-treated for one day in the dark, but this was two times higher than the activity measured at 20.5% O_2. Increasing the dark treatment to 2 days accentuated this effect

Figure 5.1: The effect of atmospheric O_2 concentration on specific nitrogenase activity during the early stages of nitrate-induced nodule senescence. Six week-old N_2-dependent plants were treated with 20mM KCl (control) or 20mM KNO_3 for 2 days prior to harvest. Root systems were assayed as described in Materials and Methods (this chapter) and each point in the Figure is the mean of 9 plants. Two-way analysis of variance indicated that there was a significant interaction between nitrate treatment prior to harvest and O_2 concentration during the acetylene reduction assay (0.05 level of significance).

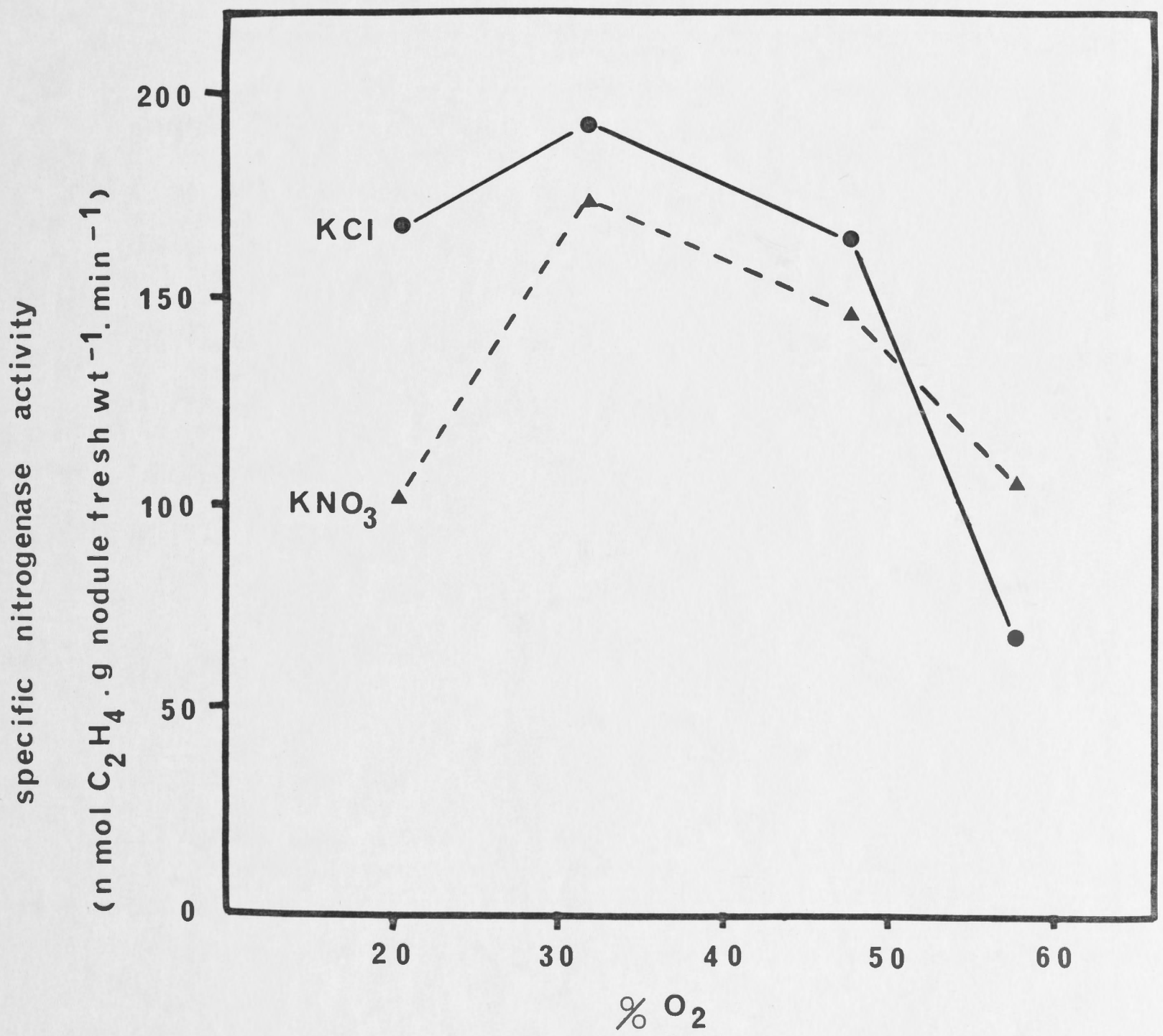
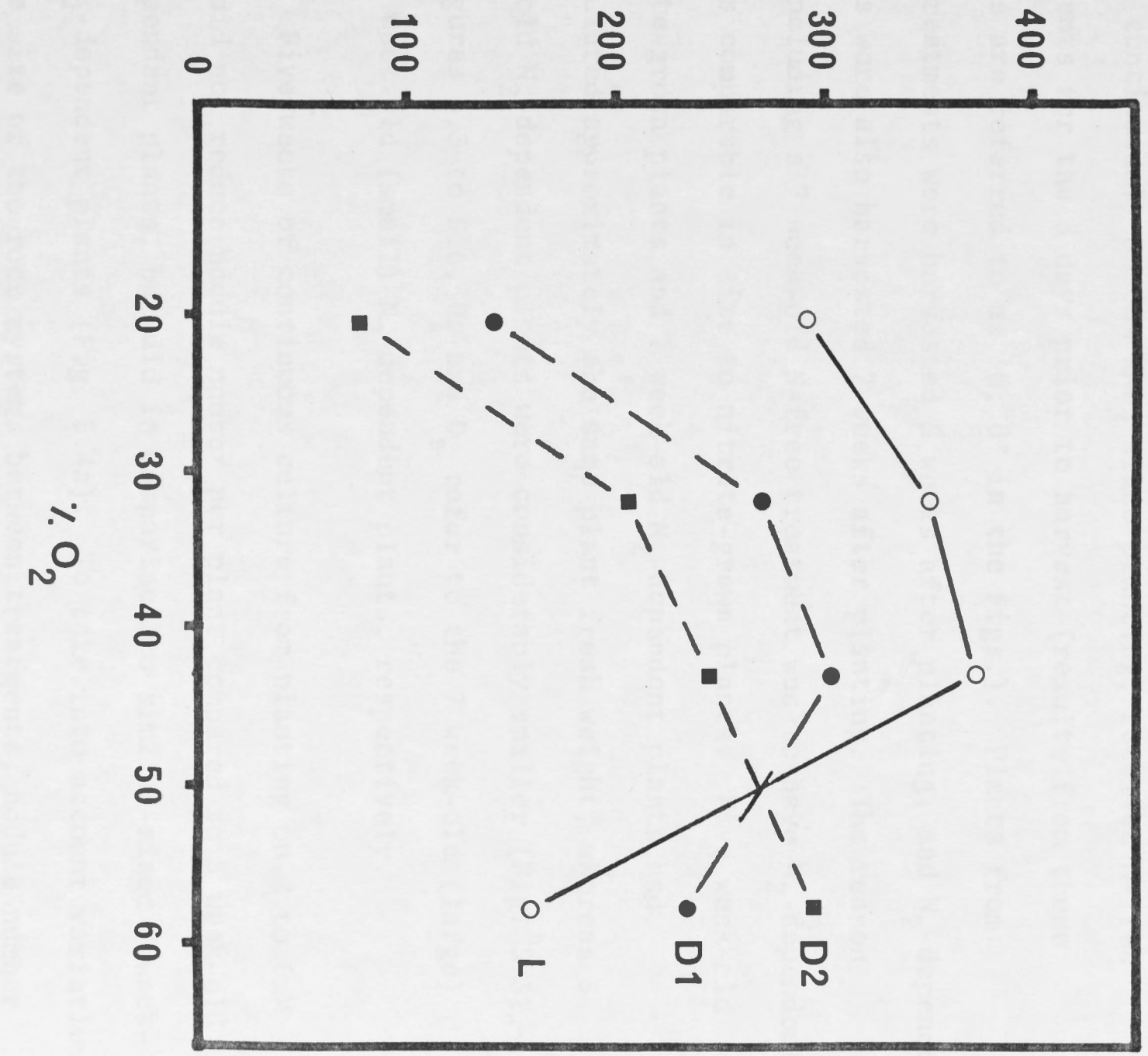

specific nitrogenase activity
(n mol C_2H_4 . g nodule fresh wt^{-1}. min^{-1})
200
150
100
50
0
KCl
KNO_3
20
30
40
50
60
% O_2

Figure 5.2: The effect of atmospheric 0_2 concentration on specific nitrogenase activity during the early stages of dark-induced nodule senescence. Six week-old N_2-dependent plants were treated with darkness for 0 (L), 1 (D1) or 2 (D2) days prior to harvest. Root systems were assayed as described in Materials and Methods (this chapter) and each point in the Figure is the mean of 6 to 12 plants. Two-way analysis of variance indicated that there was a significant interaction between dark treatment prior to harvest and 0_2 concentration during the acetylene reduction assay (0.05 level of significance).


specific nitrogenase activity
(n mol C_2H_4 . g nodule FW^{-1} . min^{-1})
0
100
200
300
400
20
30
40
50
60
% O_2
L
D1
D2

and the optimum specific nitrogenase activity, measured at 58% 0_2, was three and one half times that measured in 20.5% 0_2 (Fig. 5.5).

5.3.3 Continuous exposure of soybean plants to nitrate. With the exception of one treatment, plants in these experiments were cultured continuously on 0, 2, 4 or 6mM KNO_3 from planting and harvested 5 or 7 weeks later. The exception was that some plants that had been grown continuously on 6mM KNO_3 since planting, received N-free nutrients for the 3 days prior to harvest (results from these plants are referred to as '6, 0' in the Figs.). Plants from all treatments were harvested 5 weeks after planting, and N_2-dependent plants were also harvested 7 weeks after planting. The reason for including a 7 week-old N-free treatment was to have N_2-dependent plants comparable in size to nitrate-grown plants. Five week-old nitrate-grown plants and 7 week-old N_2-dependent plants had accumulated approximately the same plant fresh weight, whereas 5 week-old N_2-dependent plants were considerably smaller (Fig. 5.3). In Figures 5.3 to 5.6, 0_1 and 0_s refer to the 7 week-old (large) and 5 week-old (small) N_2-dependent plants, respectively.

Five weeks of continuous culture from planting on 2 to 6mM KNO_3 did not reduce nodule number per plant compared to 5 week-old N_2-dependent plants, but did in comparison to similar-sized 7 week-old N_2-dependent plants (Fig. 5.4a). To take into account variation in the size of the root systems between treatments, nodule number was also expressed per unit of root fresh weight (Fig. 5.4b). The 4 and 6mM KNO_3 treatments caused a significant inhibition of nodule number per g root fresh weight, whereas plants from the 2mM KNO_3 treatment were comparable to similar-aged N_2-dependent plants, but were significantly inhibited in this parameter when compared to similar-sized N_2-dependent plants (Fig. 5.4b). All

Figure 5.3: The effect of continuous nitrate supply on plant fresh weight accumulation. Each column represents the mean of 16 plants.

Key: '0_1' = plants were cultured on N-free nutrients and were harvested 7 weeks after planting.

'0_s' = plants were cultured on N-free nutrients and were harvested 5 weeks after planting.

'2', '4' and '6' = plants were cultured on 2, 4 and 6mM KNO_3, respectively, and were harvested 5 weeks after planting.

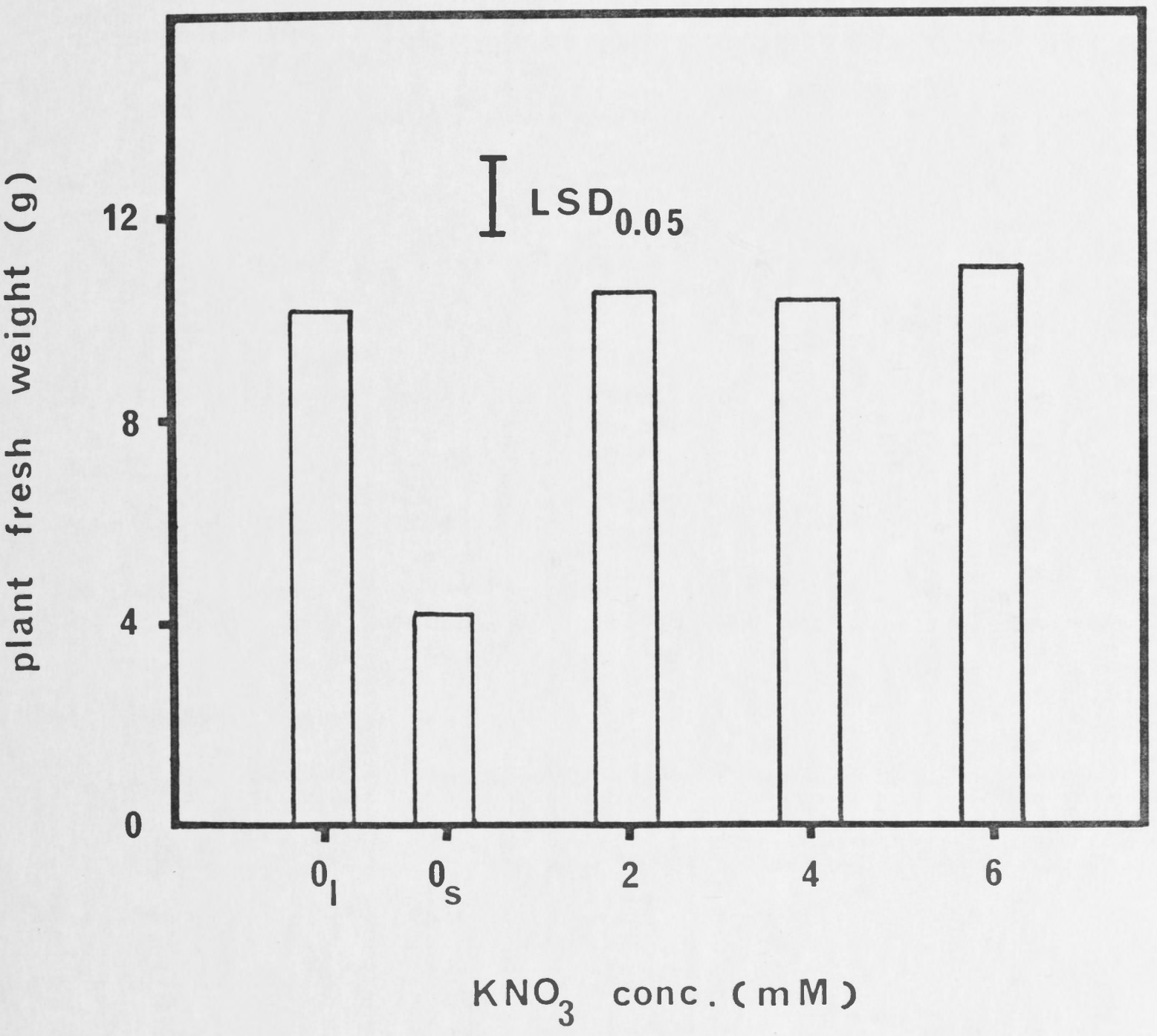

plant fresh weight (g)
12
8
4
0
LSD0.05
0l
0s
2
4
6
KNO3 conc. (mM)

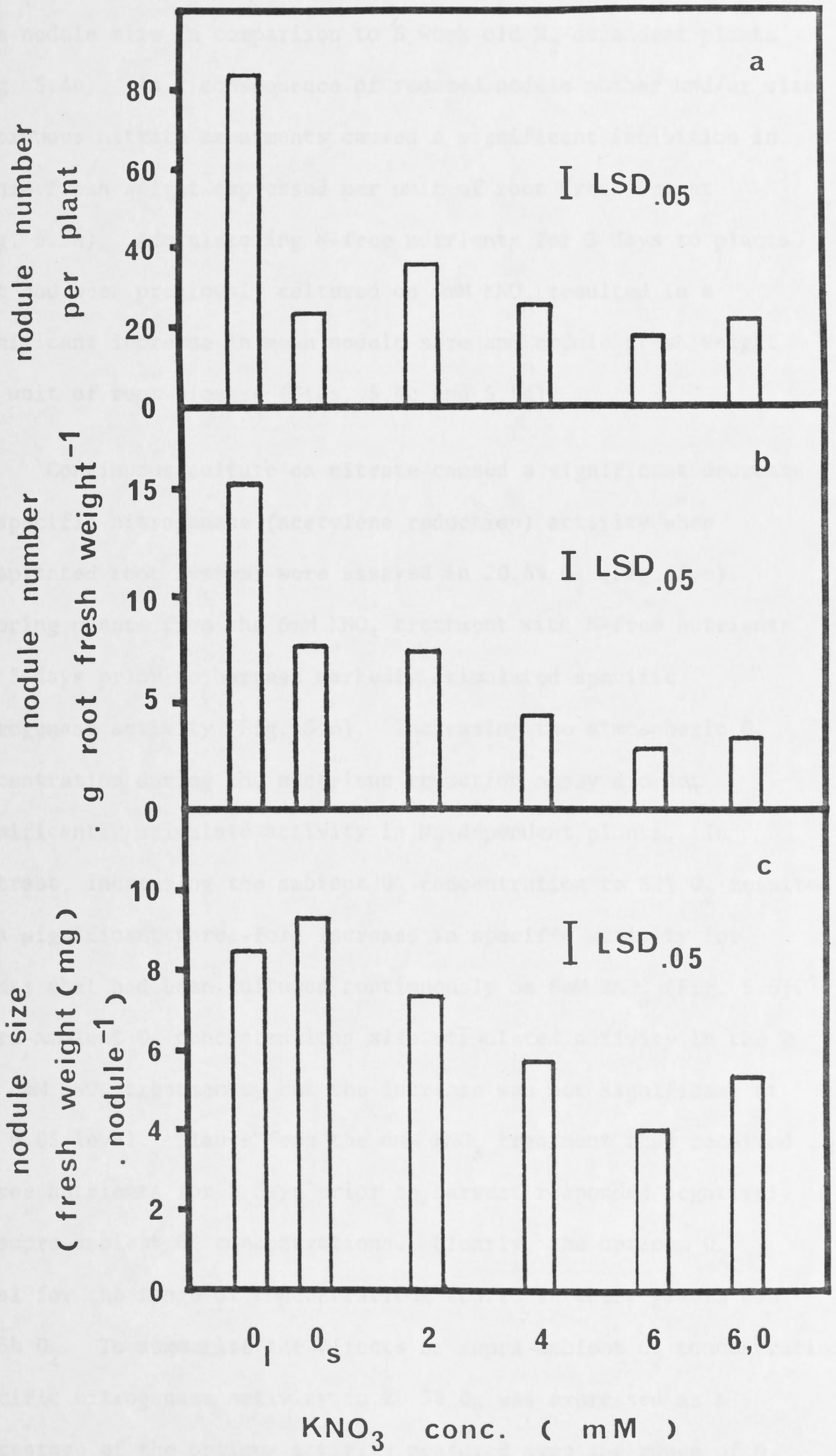
Figure 5.4: The effect of continuous nitrate supply on nodule number and nodule size. Each column represents the mean of 16 plants.

Key: '0_1' = plants were cultured on N-free nutrients and were harvested 7 weeks after planting.

'0_s' = plants were cultured on N-free nutrients and were harvested 5 weeks after planting.

'2', '4' and '6' = plants were cultured on 2, 4 and 6mM KNO_3, respectively, and were harvested 5 weeks after planting.

'6, 0' = plants were cultured on 6mM KNO_3 from day 0 to day 32 and then received N-free nutrients for the final 3 days prior to harvest.


a
b
c
nodule number per plant
nodule number . g root fresh weight^{-1}
nodule size (fresh weight (mg) . nodule^{-1})
LSD.05
LSD.05
LSD.05
80
60
40
20
0
15
10
5
0
10
8
6
4
2
0
0$_I$
0$_S$
2
4
6
6,0
KNO$_3$ conc. (mM)

continuous nitrate treatments caused a significant decrease in mean nodule size in comparison to 5 week-old N_2-dependent plants (Fig. 5.4c). As a consequence of reduced nodule number and/or size, continuous nitrate treatments caused a significant inhibition in nodule fresh weight expressed per unit of root fresh weight (Fig. 5.5a). Administering N-free nutrients for 3 days to plants that had been previously cultured on 6mM KNO_3 resulted in a significant increase in mean nodule size and nodule fresh weight per unit of root biomass (Figs. 5.4c and 5.5a).

Continuous culture on nitrate caused a significant decrease in specific nitrogenase (acetylene reduction) activity when decapitated root systems were assayed in 20.5% O_2 (Fig. 5.6). Watering plants from the 6mM KNO_3 treatment with N-free nutrients for 3 days prior to harvest markedly stimulated specific nitrogenase activity (Fig. 5.6). Increasing the atmospheric O_2 concentration during the acetylene reduction assay did not significantly stimulate activity in N_2-dependent plants. In contrast, increasing the ambient O_2 concentration to 32% O_2 resulted in a significant three-fold increase in specific activity for plants that had been cultured continuously on 6mM KNO_3 (Fig. 5.6). Supra-ambient O_2 concentrations also stimulated activity in the 2 and 4mM KNO_3 treatments, but the increase was not significant at the 0.05 level. Plants from the 6mM KNO_3 treatment that received N-free nutrients for 3 days prior to harvest responded negatively to supra-ambient O_2 concentrations. Clearly, the optimum O_2 level for the range of concentrations tested in these plants was 20.5% O_2. To summarize the effects of supra-ambient O_2 concentrations, specific nitrogenase activity in 20.5% O_2 was expressed as a percentage of the optimum activity measured over the range of O_2 concentrations tested (Fig. 5.5b).

Figure 5.5: The effect of continuous nitrate supply on nodule fresh weight and specific nitrogenase activity. a) Nodule fresh weight (mg) is expressed per g of root fresh weight; each column represents the mean of 16 plants. b) Specific nitrogenase (acetylene reduction) activity was measured in air and in various supra-ambient O_2 concentrations, and the activity measured in air is expressed as a percentage of the maximum activity measured over the range of O_2 concentrations tested (see Fig. 5.6). Each column represents the mean of 9 plants. The nitrate culture regimes are described in Figure 5.4.

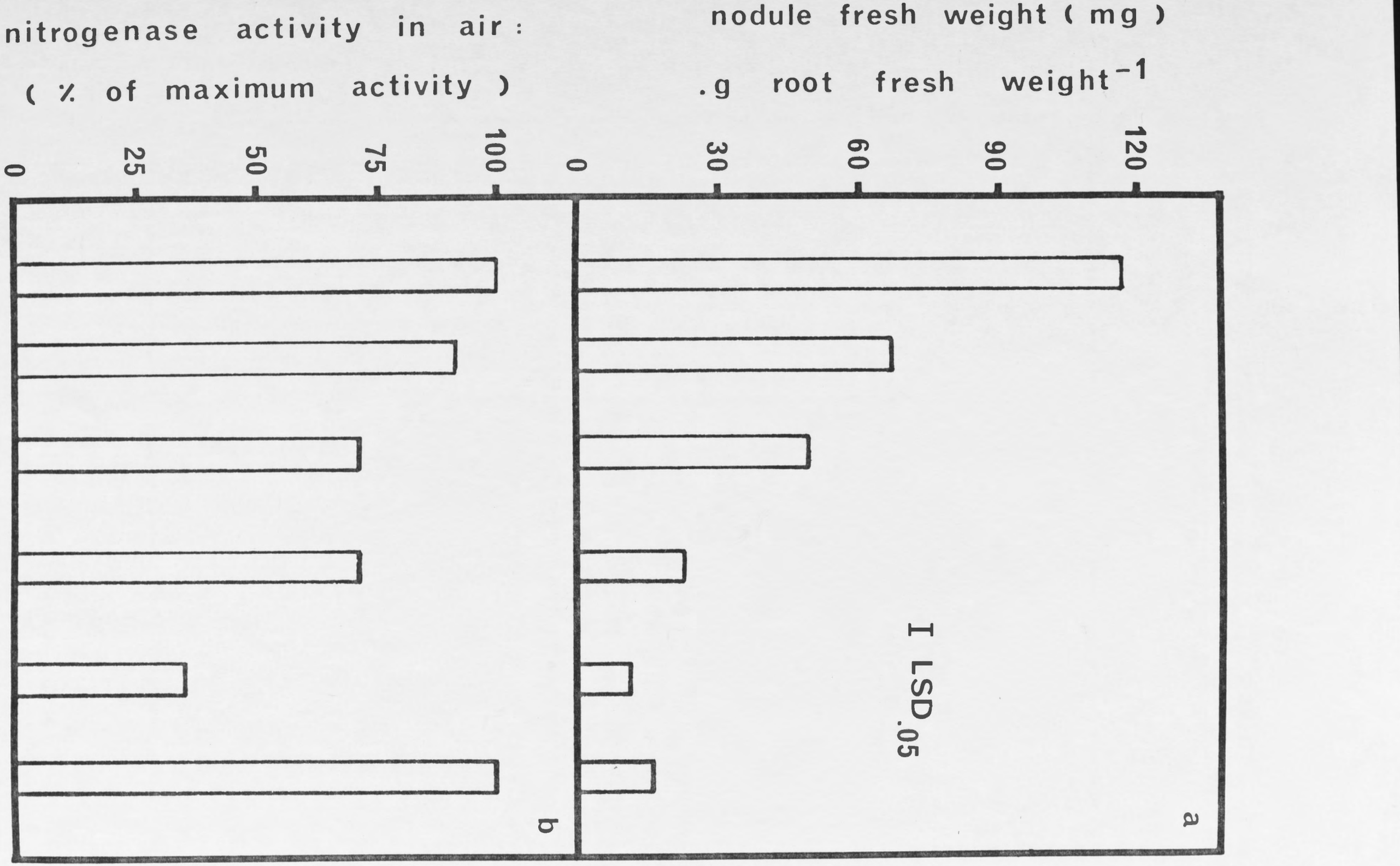
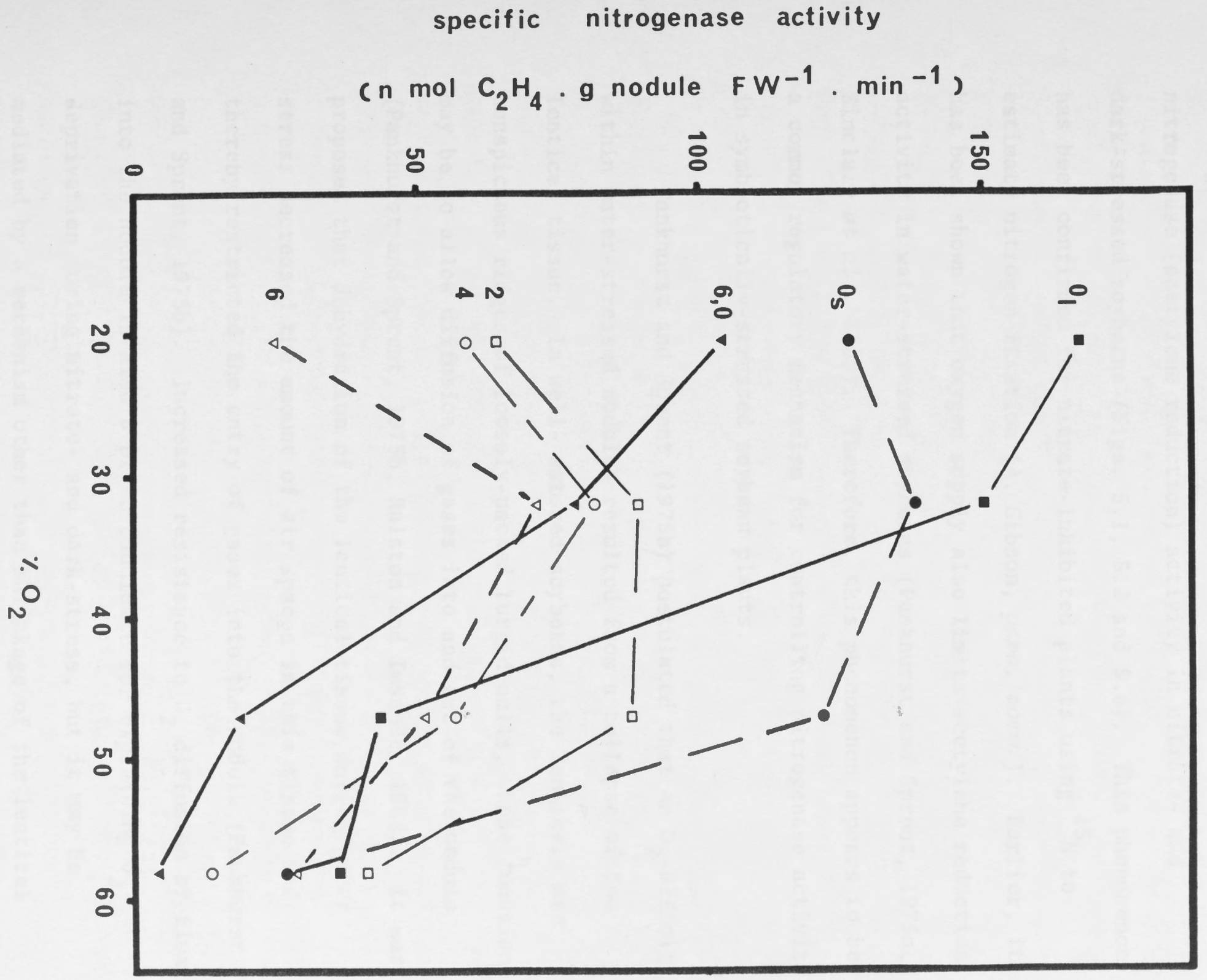

nitrogenase activity in air :
(% of maximum activity)
0
25
50
75
100
nodule fresh weight (mg)
.g root fresh weight^{-1}
0
30
60
90
120
I_0
S_0
2
4
6
6;0
KNO$_3$ conc. (mM)
a
b
I LSD$_{.05}$

Figure 5.6: The effect of continuous nitrate treatment and supra-ambient O_2 concentrations on specific nitrogenase activity. The nitrate culture regimes ('0_l', '0_s', '2', '4', '6' and '6, 0') are described in Figure 5.4 and root systems were assayed for acetylene reduction activity as described in Materials and Methods (this chapter). Each point in the Figure is the mean of 9 plants.


specific nitrogenase activity
(n mol C_2H_4 . g nodule FW^{-1} . min^{-1})
0
50
100
150
20
30
40
50
60
% O_2
6
4
2
6,0
O_S
O_I

5.4 Discussion

The results presented in this chapter clearly illustrated that O_2 supply to the nodule is a major factor limiting specific nitrogenase (acetylene reduction) activity in nitrate- and dark-stressed soybeans (Figs. 5.1, 5.2 and 5.6). This phenomenon has been confirmed for nitrate-inhibited plants using ^{15}N to estimate nitrogen fixation (A. Gibson, *pers. comm.*). Earlier, it has been shown that oxygen supply also limits acetylene reduction activity in water-stressed soybeans (Pankhurst and Sprent, 1975a; Sinclair *et al.*, 1985). Therefore, this phenomenon appears to be a common regulatory mechanism for controlling nitrogenase activity in symbiotically-stressed soybean plants.

Pankhurst and Sprent (1975b) postulated that an O_2-deficit within water-stressed nodules resulted from a collapse of the lenticel tissue. In well- watered soybeans, the lenticels are conspicuous ridges of loosely-packed turgid cells, whose function may be to allow diffusion of gases into and out of the nodule (Pankhurst and Sprent, 1975b; Ralston and Imsande, 1982). It was proposed that dehydration of the lenticel tissue during water stress decreased the amount of air spaces in this tissue and thereby restricted the entry of gases into the nodule (Pankhurst and Sprent, 1975b). Increased resistance to O_2 diffusion or flux into the nodule is also a prime candidate for explaining O_2 deprivation during nitrate- and dark-stress, but it may be mediated by a mechanism other than shrinkage of the lenticel tissue. For example, the nodule cortex in general (Tjepkema and Yocum, 1974; Sinclair and Goudriaan, 1981) may be less

permeable to oxygen diffusion or the capacity of leghemoglobin to facilitate O_2 flux to the bacteroids may be obstructed in stressed soybean plants. An alternative hypothesis to decreased O_2 uptake by the nodule is that nitrate and/or dark treatments cause a redistribution of carbon sources in the nodule and consequently precipitate a decrease in respiratory efficiency; i.e. increased O_2 consumption per N_2 (C_2H_2) reduced. Recently, it has been demonstrated that isolated soybean bacteroids consume varying amounts of O_2 per C_2H_2 reduced, depending on the carbon source supplied during the assay (McNeil *et al.*, 1984). Of course, there may be more than one factor contributing to the O_2 deprivation response and the mechanism may vary with the type of stress.

Since the pertinent physiological processes that are ultimately responsible for O_2 deprivation are not known at this stage, one can only speculate in reconciling the results presented in this chapter with other reports in the literature. The reduction of nitrate and N_2 are energy-dependent and carbohydrate deprivation has been implicated to be responsible for nitrate (Oghoghorie and Pate, 1971; Gibson and Pagan, 1977), as well as dark-induced (Schweitzer and Harper, 1980), inhibition of specific nitrogenase activity. Studies on dark-induced nodule senescence in soybean have shown that loss of nitrogenase activity was not reflected in sugar and starch concentrations in the nodule (Schweitzer and Harper, 1980), although total nonstructural carbohydrates did decline (Schweitzer and Harper, 1980) and bacteroids from dark-inhibited plants were apparently deprived of endogenous energy reserves that are capable of supporting nitrogenase activity (Chapter 4). There was no convincing evidence for nodule

(Streeter, 1981) or bacteroid (Chapter 4) carbohydrate deprivation in nitrate-treated soybeans. A common reported feature of nitrate- (Streeter, 1981) and dark- (Schweitzer and Harper, 1980) inhibited soybean plants is that sugar concentrations are decreased in the shoot tissue. Interestingly, the metabolic rate of the shoot tissue directly regulates nitrogenase (acetylene reduction) activity in soybean, since short term (5h) decreases in the temperature around the shoots has been demonstrated to cause a decline in nodule activity (Schweitzer and Harper, 1980). Perhaps the level of certain carbohydrates in the shoot are important in mediating oxygen deprivation in soybean nodules during culture on nitrate or in the dark. An alternative hypothesis to carbohydrate deprivation during culture on nitrate is that products of nitrate reduction are responsible for inhibition of nitrogenase activity (Streeter, 1982a). Both nitrite (Rigaud and Puppo, 1977) and a nitrosyl radicle (Maskall *et al.*, 1977) are plausible candidates for inhibiting the function of leghemoglobin *in vivo* (Chapter 4). Acetylene saturation studies on nodule activity (Denison *et al.*, 1983) and electron-paramagnetic-resonance studies of leghemoglobin *in vivo* (Maskall *et al.*, 1977; C.A. Appleby, *pers. comm.*) should make it possible to distinguish between general resistance to gaseous diffusion and leghemoglobin-mediated O_2 deprivation in the nodules.

Although O_2 supply is a major factor limiting nitrogenase activity in the early stages of nitrate- and dark-induced nodule senescence (Figs. 5.1 and 5.2), other mechanisms appeared to be operative in the later stages of inhibition. As described in Chapter 4 and alluded to in the above discussion, treatment of

N_2-dependent plants in the dark resulted in a substantial decline in the apparent level of endogenous bacteroid energy reserves that were capable of supporting nitrogenase activity. And although there was no evidence for bacteroid carbohydrate deprivation during nitrate-induced nodule senescence, the integrity of isolated bacteroids was affected after 4 to 6 days of culture on nitrate (Chapter 4). Besides isolated bacteroid characteristics, the amounts of leghemoglobin and other proteins eventually declined during nitrate- and dark-induced nodule senescence (Pfeiffer *et al.*, 1983; Chapter 4; Schuller *et al.*, 1985). The relationship between 0_2 deprivation and these other phenomena is unknown at this stage, except that 0_2 limitation appears to preempt other detectable changes that would affect nodule functioning.

Regulation of oxygen supply within the nodule was not restricted to nodule senescence and 0_2 supply was also a major factor limiting nitrogenase (acetylene reduction) activity in plants that had been grown continuously on nitrate (Fig. 5.6). Consistent with this regulatory model, watering nitrate-grown plants with N-free nutrients for 3 days prior to harvest caused a significantly different response of nitrogenase activity to changes in the ambient 0_2 concentration during the acetylene reduction assay. Optimum specific nitrogenase activity for these plants was measured in 20.5% 0_2 and higher 0_2 concentrations inhibited nodule activity (Fig. 5.6). Transfer of plants from nitrate to N-free culture conditions for 3 days prior to harvest also resulted in a significant increase in nodule growth (Figs. 5.4c and 5.5a). It has been reported that exposure of soybean roots to low 0_2 tensions caused a curtailment in nodule development, but not in nodule initiation (Bond, 1950). Therefore, it is possible that an 0_2 deficit in the nodules of

nitrate-grown plants restricts nodule growth as well as specific nitrogenase activity. This possibility is being further investigated.

CHAPTER SIX

ISOLATION AND PROPERTIES OF NOVEL SOYBEAN (*Glycine max* (L.) Merr.) MUTANTS THAT NODULATE IN THE PRESENCE OF HIGH NITRATE CONCENTRATIONS.

6.1 Introduction

Modern techniques in molecular biology, such as site-directed mutagenesis, have enabled the isolation of many *Rhizobium* mutants that have led to an increased understanding of the heritable attributes of the microsymbiont required for a functional symbiosis (Pühler *et al.*, 1984). In contrast, only a few heritable host factors have been reported to influence the extent of symbiosis in legumes (Nutman, 1981). The most striking variation has resulted in decreased nodulation or absence of nodulation (Nutman, 1981). Nevertheless, variants within existing germplasms with increased nodulation have been demonstrated in some species (Nutman, 1953; Gelin and Blixt, 1964; Mytton and Jones, 1971; Nutman *et al.*, 1971; Duhigg *et al.*, 1978; Heichel *et al.*, 1984; Imsande, 1984). These promising variants with enhanced nodulation have previously all been selected in the absence of nitrate (Gelin and Blixt, 1964; Heichel *et al.*, 1984).

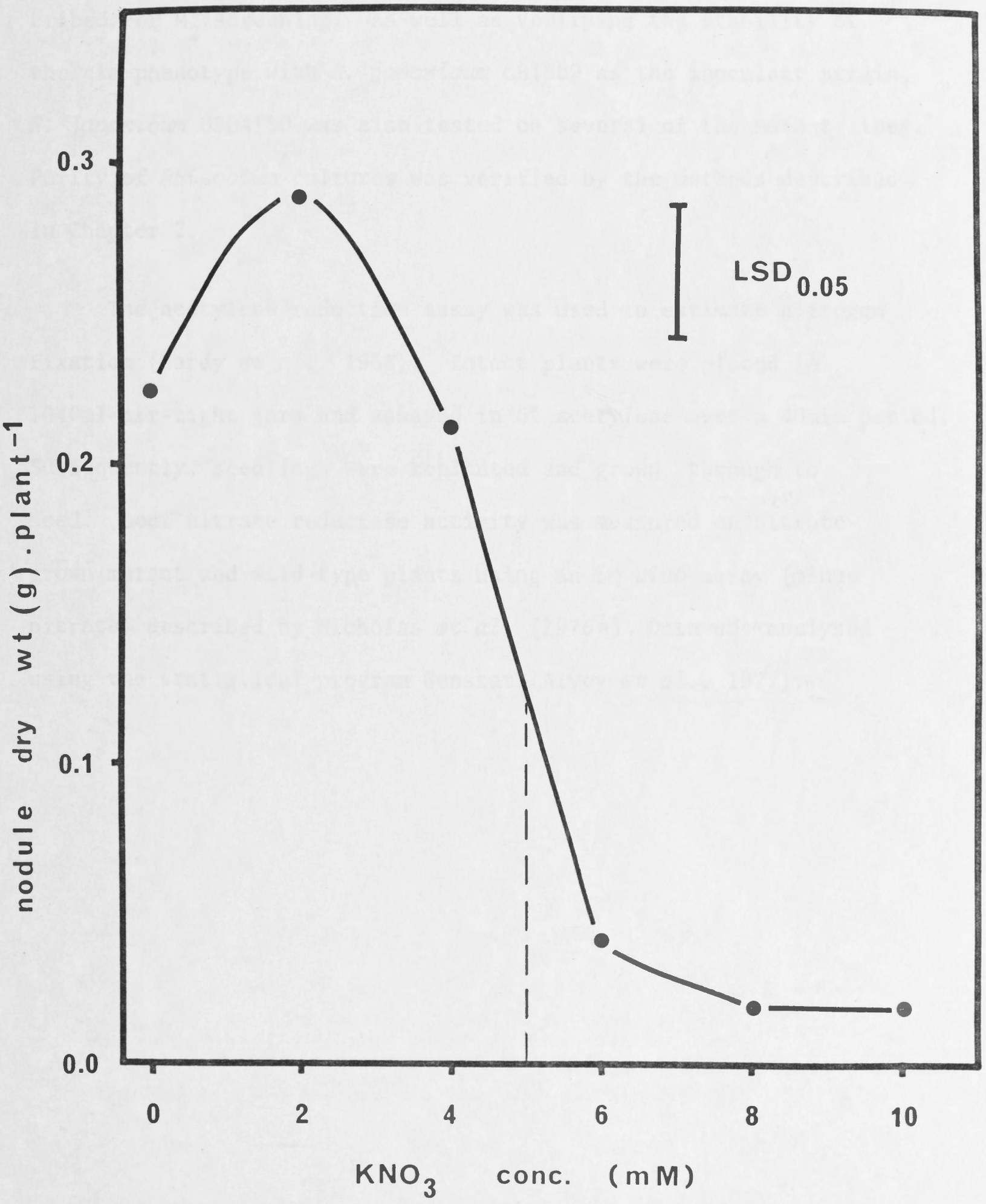
Consistent with the trend of greater emphasis on bacterial genetics rather than host genetics, attempts to circumvent nitrate inhibition by genetic manipulation of the symbionts have been confined to *Rhizobium* (Gibson and Pagan, 1977; McNeil, 1982; Streeter, 1982a). These studies have implied that the host genome is responsible for this regulatory phenomenon. However, to date only minor differences in symbiotic tolerance to nitrate have been demonstrated between host species (Carroll *et al.*, 1984; Harper and Gibson, 1984b) and between cultivars within a species (Carroll *et al.*, 1984; Harper and Gibson 1984a; Gibson and Harper, 1985).

6.2 Materials and Methods

Seeds of soybean cv Bragg were mutagenized with EMS, sodium azide (NaN_3) or gamma-rays (Chapter 3). EMS was the most efficient mutagen for generating chlorophyll-deficient mutants (Chapter 3) and plants from EMS-mutagenized populations were used in screening for mutants that nodulate in the presence of nitrate. Screening was carried out on the M_2 generation.

For *nts* mutant selection, 12 seeds from each M_2 family were planted at 2cm depth in a pot of river sand (25cm diam., 25cm height) and inoculated with *Rhizobium japonicum* strain CB1809 (=USDA136). The plants were cultured in the presence of nitrate for 5 to 7 weeks, then carefully removed from the sand and visually screened for the extent of nodulation. Pots were initially watered with nutrient solution three times per week increasing to daily additions as the demand for water increased with growth. The nutrient solution was the same as that used by Herridge (1977), except that all nutrients other than $CaCl_2$ and KNO_3 were administered at one-quarter strength for the first 2 weeks. The nitrate concentration in the nutrient solution was 5mM throughout. To minimize fluctuations in nitrate levels, the pots were flushed with nutrient solution (1.4 litres) at each watering. Five milli-molar KNO_3 was chosen on the basis of a nitrate concentration vs nodule dry weight profile obtained with the parent cultivar Bragg (Fig. 6.1). The plants were grown outside during summer and in glasshouses during winter, spring and autumn. The plants were grown at a latitude of 37^{o} 17' S with mean summer minima and maxima temperatures of $12.4^{o}C$ and $26.9^{o}C$ respectively. Glasshouse temperature were held between

Figure 6.1: Effect of nitrate concentration on nodule dry weight in parent cultivar Bragg. Plants were harvested 7 weeks after planting. Inoculant was *R. japonicum* strain CB1809.


0.3
0.2
0.1
0.0
nodule dry wt (g).plant-1
LSD0.05
0
2
4
6
8
10
KNO3 conc. (mM)

14°C and 30°C. Subsequent generations of *nts* mutants were tested under the conditions described above. However, smaller pots (20cm diam., 20cm height) were occasionally used instead of the pots described for M_2 screening. As well as verifying the stability of the *nts* phenotype with *R. japonicum* CB1809 as the inoculant strain, *R. japonicum* USDA110 was also tested on several of the mutant lines. Purity of *Rhizobium* cultures was verified by the methods described in Chapter 2.

The acetylene reduction assay was used to estimate nitrogen fixation (Hardy *et al.*, 1968). Intact plants were placed in 1040ml air-tight jars and assayed in 6% acetylene over a 40min period. Subsequently, seedlings were replanted and grown through to seed. Leaf nitrate reductase activity was measured on nitrate-grown mutant and wild-type plants using an *in vivo* assay (minus nitrate) described by Nicholas *et al.* (1976a). Data was analysed using the statistical program Genstat (Alvey *et al.*, 1977).

6.3 Results

When two thousand five hundred families (approximately 25,000 M_2 seedlings) were screened for nodulation on nitrate, fifteen of these families segregated for the *nts* phenotype. These variants had significantly increased nodulation in the presence of 5mM KNO_3 compared with wild-type siblings and the parent cultivar (Table 6.1). Expression of the *nts* character was not confined to mutant plants inoculated with *R. japonicum* CB1809 (= USDA136). *R. japonicum* USDA110 also elicited the *nts* phenotype in the six mutants so far tested (see Table 6.1). Stable inheritance of the *nts* character has been demonstrated through to at least the M_3 generation in the ten mutant families listed in Table 6.1. Many other selections showed marginally increased nodulation, but these will not be further discussed.

6.3.1 Nodulation of *nts* mutants. Table 6.1 shows nodule number for *nts* mutants and wild-type cultured on 5mM KNO_3 for 5-7 weeks. Nodule number per plant was substantially higher in the mutants than in the wild type, as was nodule fresh weight. After 6-9 weeks culture on 5mM KNO_3, *nts* mutants had 5 to 20 times the nodule mass of wild-type plants (Table 6.2).

Families *nts*382 and *nts*1116 were studied in more detail than any other of the mutant families. These two mutants were compared with Bragg for nodulation in the presence and absence of nitrate. Not only did *nts*1116 and *nts*382 nodulate more than the wild-type during culture on nitrate (Table 6.1), but there was also a striking difference between these mutants and the wild type in the absence of nitrate (Fig. 6.2, Table 6.3).

Table 6.1: Nodule number for *nts* and wild-type cultured on 5mM KNO_3 for 5-8 weeks. Unless noted, the data are for M_2 plants and wild-type refers to wild-type siblings of respective *nts* mutants. The inoculant strain for this data was *R. japonicum* CB1809 (=USDA136), however, *R. japonicum* USDA110 also elicited the *nts* phenotype in the six mutant lines so far tested (see d in Table).

Table 6.1

Selected family	nodule number . plant^{-1} (± S.D.)	
	nts mutants	wild-type
382[d]	146 ± 71	26 ± 11
1007[d]	179 ± 39	13 ± 4
1116[b,d]	79 ± 60	19 ± 7[a]
246[b]	115 ± 74	8 ± 2
733[d]	213 ± 177	18 ± 10
183[d]	269 ± 70	19 ± 8
97[c]	120	32 ± 7
501[c,d]	251	19 ± 8
2062	233 ± 45	34 ± 19
2264[b]	409 ± 148	12 ± 6[a]

[a] parent cultivar Bragg

[b] data from M_3 plants

[c] data for *nts* mutants from one plant only

[d] *nts* phenotype was also elicited using *R. japonicum* USDA110 as the inoculant strain.

Table 6.2: Nodule fresh weight for *nts* mutants and wild-type cultured on 5mM KNO_3 for 6-9 weeks. Unless noted, the data are for M_3 plants. The inoculant strain was *R. japonicum* CB1809 (=USDA136).

Table 6.2

Selected family	nodule fresh weight (mg). plant^{-1} (± S.D.)	
	nts mutants	wild-type[c]
1007	1196 ± 377	62 ± 25[d]
246[a]	1537 ± 647	198 ± 134
733	1086 ± 349	86 ± 53
183	1715 ± 411	187 ± 91[d]
501	445 ± 252	64 ± 35
2062[b]	1929 ± 391	176 ± 118[d]
2264	941 ± 307	86 ± 53

[a,b] data from M_4 and M_2 plants respectively

[c] unless otherwise noted, wild-type refers to parent cultivar Bragg

[d] non-*nts* siblings of respective mutants were used as wild-type controls.

Figure 6.2: Nodule development in *nts*1116 and wild-type plants grown in the presence and absence of 2.5mM KNO_3. Nodule number (A) and nodule fresh weight (B) are expressed per plant. The inoculant strain was *R. japonicum* CB1809 (=USDA136) and the pots (20cm diam., 20cm height) were watered daily with nutrient solution. Plants were harvested 45 days after planting. Each column in the Figure is the mean of 4 to 14 plants.

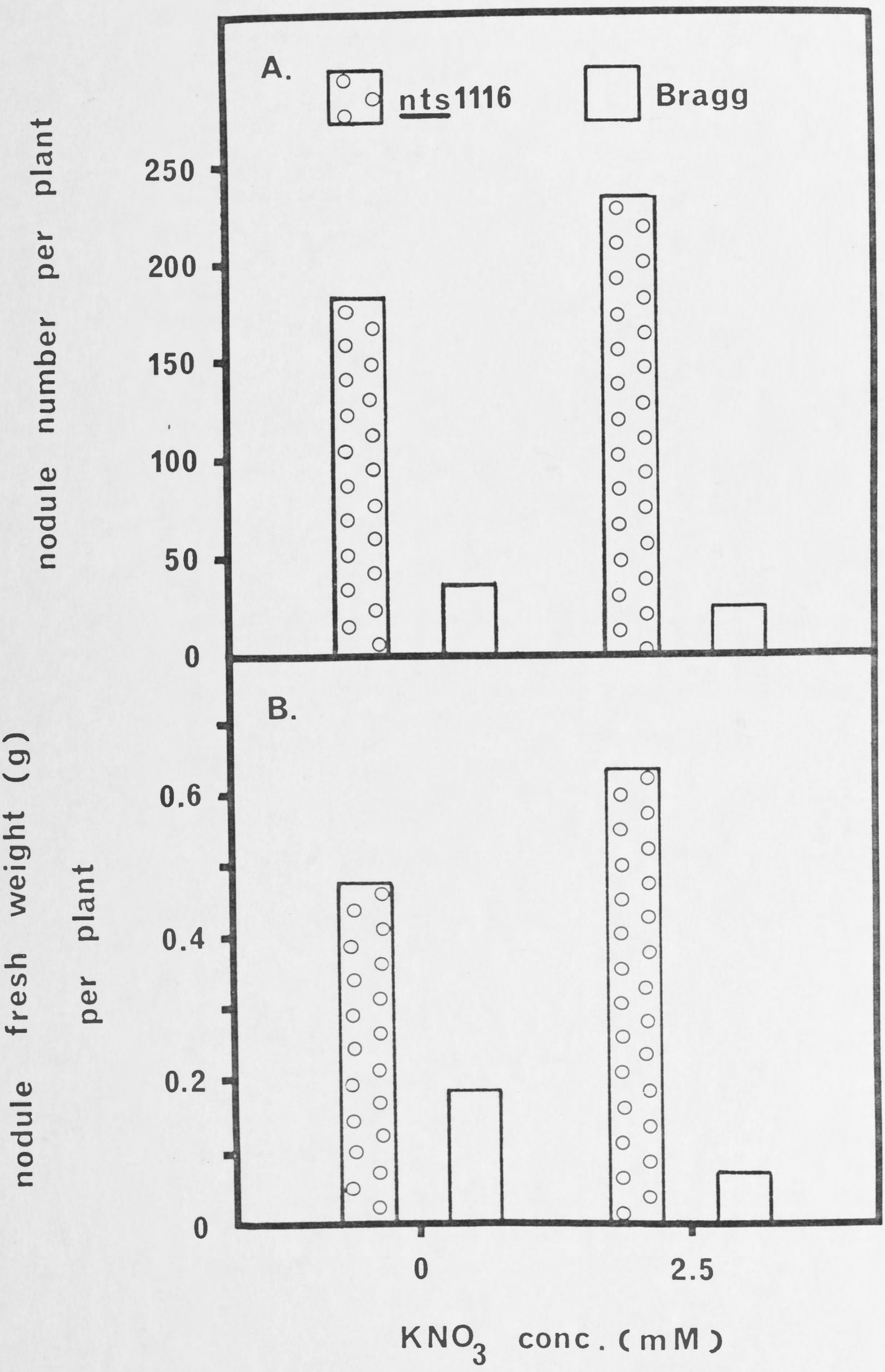

A.
nts1116
Bragg
nodule number per plant
250
200
150
100
50
0
B.
nodule fresh weight (g) per plant
0.6
0.4
0.2
0
0
2.5
KNO3 conc. (mM)

Table 6.3: Plant fresh weight and nodule development in *nts*382 in the presence and absence of 5.5mM nitrate. The inoculant strain was *R. japonicum* USDA110 and the pots were watered daily with nutrient solution. Plants were harvested at 31 and 64 days after planting. Each entry in the Table for 31 day-old plants represents the mean of 4 to 5 plants, whereas for the older plants each entry is the mean of 9 to 28 plants. The effects of plant genotype and nitrate treatment for the different-aged plants were analysed using two-way analysis of variance.

Table 6.3:

Days after planting	Plant line	KNO_3 conc. (mM)	Plant fresh weight (g)	Nodule no. per plant	mg. nodule fresh weight per plant
31[a]	*nts*382	0	2.31[c]	95[e]	215[e]
	Bragg	0	2.17	3	8
	*nts*382	5.5	3.64	35	74
	Bragg	5.5	3.12	2	4
64[b]	*nts*382	0	11.8[d]	431[e]	1583[e]
	Bragg	0	21.1	69	886
	*nts*382	5.5	28.5	414	1886
	Bragg	5.5	40.5	29	174

[a] plants were at the primary leaf-first trifoliolate leaf stage of development

[b] N_2-dependent plants had four to five fully open trifoliolate leaves, whereas nitrate-fed plants had six to nine trifoliolate leaves

[c] significant nitrate treatment effect on 31 day-old plants

[d] significant nitrate treatment and genotype effects on 64 day-old plants

[e] nitrate treatment, genotype and interaction effects were significant.

After 45 days culture without nitrate, *nts*1116 plants had five times as many nodules as parent cultivar Bragg; with nitrate present, there were nine times as many nodules on *nts*1116 plants (Fig. 6.2A). Two-way analysis of variance of the data in Fig. 6.2A showed that there was a significant genotype effect, but no significant effect of nitrate on nodule number per plant for *nts*1116 or for Bragg. Consistent with reports in the literature (Streeter, 1981) nodule growth was more sensitive to nitrate inhibition than was nodule initiation in wild-type soybean (Fig. 6.2). Wild-type plants grown on nitrate had only 40% of the nodule mass of N_2-dependent wild-type plants (Fig. 6.2B). Nodule mass was consistently higher in *nts*1116 than in Bragg (Fig. 6.2B). In the absence of nitrate, *nts*1116 plants had two and one-half times the nodule mass of Bragg plants and culture on nitrate accentuated the difference with *nts*1116 having 9 times the nodule mass of the wild-type (Fig. 6.2B). In contrast to Bragg, supplementing the nutrient solution with 2.5mM KNO_3 throughout the 45 days of culture did not inhibit nodule growth per plant in *nts*1116 (Fig. 6.2B).

A similar trend was seen for *nts*382 (Table 6.3). Winter-grown Bragg and *nts*382 plants were harvested at 31 and 64 days after planting. In the early stages of nodule development, *nts*382 plants had a significantly higher nodule number and nodule fresh weight than did wild-type plants. Irrespective of nitrate supplementation, considerable differences in nodulation were also observed 64 days after planting. In the absence of nitrate, *nts*382 had six times the nodule number and twice the nodule fresh weight of the wild-type and, as was the case for *nts*1116, administering nitrate during growth increased the difference in nodulation between *nts*382 and Bragg. Figure 6.3 shows the root systems of *nts*382 and wild-type Bragg

Figure 6.3: Bragg and *nts*382 plants after 4 weeks of culture on 0mM KNO_3 (5.5mM KCl control) (A) and on 5.5mM KNO_3 (B). Inoculant was *R. japonicum* strain CB1809.

A

B

plants that had been cultured on 0mM KNO_3 (5.5mM KCl control) and 5.5mM KNO_3 for 4 weeks. Figure 6.3 clearly illustrates the striking enhancement in nodulation observed in this mutant.

6.3.2 Acetylene reduction activity for *nts* mutants. Four weeks after planting and culture without nitrate, *nts*382 had approximately the same nitrogenase activity per plant as the parent cultivar Bragg (Table 6.4). In contrast, *nts*382 plants cultured on 2.75mM KNO_3 had ten times the nitrogenase activity of wild-type plants cultured under identical conditions. Similar results were obtained for other mutants cultured on 5mM nitrate. For example, 9 week-old *nts*1116 plants had seven times the activity of the wild-type. In other experiments, *nts*2264 and *nts*1007 plants had eight times the nitrogenase activity of wild-type plants (Table 6.4).

As stated above, N_2-dependent *nts*382 plants had a greater nodule mass than did the wild-type controls. This implies that the equality of N_2-dependent *nts*382 and wild-type plants in reducing acetylene (Table 6.4) resulted from decreased specific nitrogenase activity (activity per unit of nodule mass) in the mutant. Indeed, in 64 day-old plants grown without nitrate, specific nitrogenase activity for *nts*382 plants was 81.9 $\pm$ 16.3 ($\pm$ S.D.) nmol C_2H_4 produced . g nodule fresh weight^{-1}. min^{-1}, compared with 338.9 $\pm$ 48.4 for wild-type Bragg (Fig. 6.4). Specific nitrogenase activity of *nts*382 plants that received 5.5mM KNO_3 throughout growth was 105.7 $\pm$ 39.6 compared with 154.7 $\pm$ 43.6 for Bragg. Clearly, the addition of 5.5mM KNO_3 to the nutrient solution did not further decrease specific nitrogenase activity in the mutant (Fig. 6.4).

Table 6.4: Nitrogenase activity by mutant and wild-type plants. Mutant (M_3 or M_4 generation) and wild-type plants were compared after culture in the presence of nitrate, and for *nts*382, a comparison was made in the absence of nitrate also. Nitrogenase (acetylene reduction) activity is expressed per plant. The inoculant strain was *R. japonicum* CB1809 (=USDA136). Each entry for nitrogenase activity in the Table is the mean of 4 to 9 plants.

Table 6.4

nts mutant	KNO_3 conc. (mM)	Nitrogenase activity (nmol C_2H_4.plant^{-1}.min^{-1}) mutant	wild-type[a]	$LSD_{.05}$
382[b]	0	17.0	19.2	N.S.[d]
	2.75	34.2 (3.5)	3.4 (1.2)	(0.8)[e]
1116[c]	5	143.7	21.4	39.0
2264[c]	5	171.9	20.5	71.6
1007[c]	5	90.1	10.9	21.3

[a] parent cultivar Bragg, except in 1007 experiment where non-*nts* 1007 siblings (wild-type phenotype) were used for comparison.

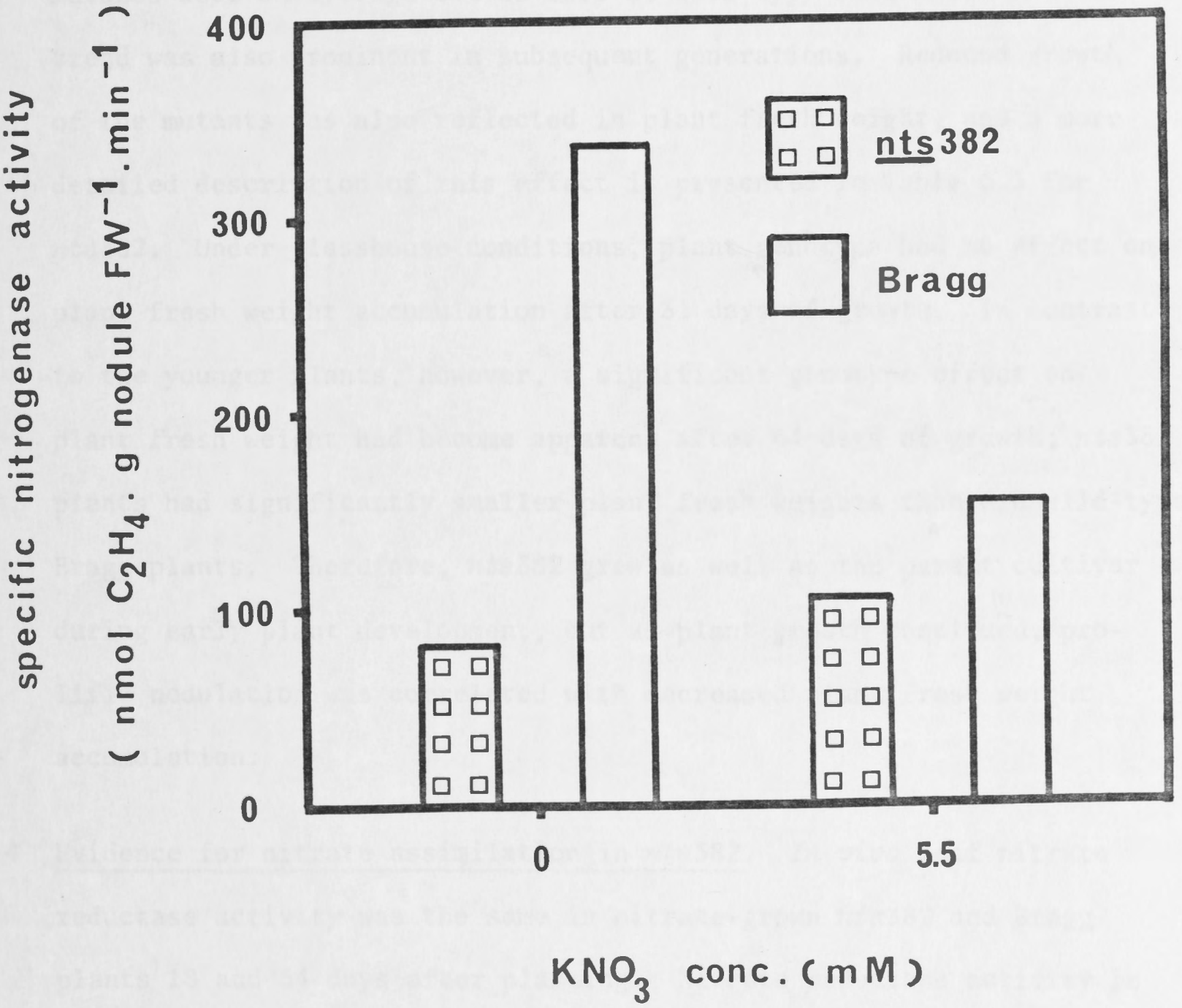
[b] harvested after 4 weeks culture in sand pots watered daily with nutrient solution

[c] harvested after culture for 9 weeks (for 1116 experiment), 8 weeks (for 2264 experiment) and 7 weeks (for 1007 experiment) in sand pots watered three times a week with 5mM KNO_3

[d] mutant and wild-type plants were not significantly different

[e] raw data required $\log_e$ transformation to satisfy assumptions for an analysis of variance; means and LSD of transformed data are shown in brackets.

Figure 6.4: Specific nitrogenase activity of Bragg and *nts*382. Plants were cultured on 0mM and 5.5mM KNO_3 for 64 days prior to harvest. Plants were inoculated with *R. japonicum* strain USDA110. Each column in the Figure is the mean of 5 to 14 plants. Two-way analysis of variance indicated that genotype, nitrate treatment and interaction effects were highly significant.


specific nitrogenase activity
(nmol C_2H_4 . g nodule FW^{-1}.min^{-1})
400
300
200
100
0
nts382
Bragg
0
5.5
KNO_3 conc. (mM)

6.3.3 Growth of *nts* mutants. Figure 6.3 illustrates that prolific nodulation in *nts*382 may have occurred to the detriment of root growth. Indeed, plants displaying the *nts* phenotype were generally smaller than wild-type non-*nts* siblings and parent cultivar Bragg. The composite data of selected M_2 *nts* mutants showed that at the time of screening (approximately 6 weeks after planting) the mutants were on average 84% as tall as wild-type siblings. This trend was also prominent in subsequent generations. Reduced growth of the mutants was also reflected in plant fresh weight, and a more detailed description of this effect is presented in Table 6.3 for *nts*382. Under glasshouse conditions, plant genotype had no effect on plant fresh weight accumulation after 31 days of growth. In contrast to the younger plants, however, a significant genotype effect on plant fresh weight had become apparent after 64 days of growth; *nts*382 plants had significantly smaller plant fresh weights than did wild-type Bragg plants. Therefore, *nts*382 grew as well as the parent cultivar during early plant development, but as plant growth continued, prolific nodulation was correlated with decreased plant fresh weight accumulation.

6.3.4 Evidence for nitrate assimilation in *nts*382. *In vivo* leaf nitrate reductase activity was the same in nitrate-grown *nts*382 and Bragg plants 18 and 64 days after planting. Nitrate reductase activity in the primary leaf was of 18 day-old plants 14.3 $\pm$ 3.0 ($\pm$ S.D.) µmol nitrite .g plant fresh weight^{-1}.h^{-1} for *nts*382 and 16.6 $\pm$ 7.2 ($\pm$ S.D.) µmol nitrite .g plant fresh weight^{-1}.h^{-1} for Bragg. Since in these experiments nitrate was not added to the assay solution, nitrite produced during the assay was derived from nitrate present in the tissue at the time of harvest.

Supplementing the nutrient solution with nitrate stimulated plant fresh weight accumulation in *nts*382 as well as in Bragg (Table 6.3). This stimulation was observed at both 31 and 64 days after planting. Clearly, *nts*382 plants can take up, reduce and assimilate nitrate.

6.4 Discussion

Despite the immense importance of the root system of plants, the literature is almost devoid of reports detailing mutations that affect root development (Gottschalk and Wolff, 1983). This study has demonstrated that *nts* (nitrate-tolerant symbiosis) mutants can be isolated from mutagenized soybean populations. The frequency of *nts* mutants correlated well with the frequency of chlorophyll-deficient mutants in the two EMS-mutagenized populations used here (Table 6.5). Comparing these results with another study (D. McNeil, *pers. comm.*), it is apparent that EMS is more efficient than gamma-rays in generating *nts* mutants in soybean (Table 6.5).

A host factor(s) is responsible for the expression of the *nts* phenotype reported here. Inheritance of the *nts* character from one soybean generation to the next has been demonstrated in the ten selected lines so far tested. All selected lines came from M_2 families that segregated for the *nts* phenotype, indicating that the mutations were a result of the mutagenesis program. Indeed, each of the 15 selections came from independent mutation events, since M_2 families were maintained separately. Preliminary phenotypic grouping indicates that there are three or perhaps four different types of mutants.

After culture on nitrate, the mutants were distinguishable from the wild-type on the basis of nodule number (Table 6.1), nodule fresh weight (Table 6.2) and nitrogenase activity (Table 6.4) per plant. Theoretically, enhanced nodulation in the presence of nitrate can result from the plant's inability to either a) ulitize nitrate, or b) regulate nodule initiation and/or nodule growth in the normal

Table 6.5: Frequency of chlorophyll-deficient (chl^-) variants and *nts* variants in M_2 soybean populations.

Table 6.5

Mutagen	Dose	Frequency of chl^- variants (A)	Frequency of *nts* variants (B)	A/B
gamma-rays	15 krad	0.6×10^{-2}	$<0.2 \times 10^{-4}$ [a]	>300
EMS	0.44%, 4h [b]	0.9×10^{-2}	3.6×10^{-4}	25
EMS	0.5%, 6h [c]	2.8×10^{-2}	1.4×10^{-3}	20

[a] no *nts* variants were found in 6,000 plants screened (D. McNeil, *pers. comm.*)

[b] EMS population 1 (see Chapter 3)

[c] EMS population 2 (see Chapter 3).

manner. Mutants *nts*382 and *nts*1116 fall into the latter category. In addition to supernodulation in the presence of nitrate, both of these mutants had enhanced nodulation in the absence of nitrate. For example, under nitrate-free conditions, 64 day-old *nts*382 plants and 45 day-old *nts*1116 plants had six and five times as many nodules as the respective Bragg controls. Furthermore, there is convincing evidence that *nts*382 can utilize nitrate. Nitrate stimulated plant fresh weight accumulation in *nts*382 to a similar extent as it did in Bragg (Table 6.3), and *nts*382 and Bragg had similar leaf nitrate reductase activities. These results showed conclusively that *nts*382 and *nts*1116 are mutants in a nodule development regulatory gene(s) and not in a gene directly associated with nitrate metabolism.

Nitrate represents a major environmental factor that controls nodulation in legumes. Besides such external regulatory factors, nodulation is internally regulated by a process that has been termed autoregulation. Effectively fixing nodules 'signal' younger root tissues to restrict further nodule development. Removal of nodules or infection by a non-fixing *Rhizobium* removes this nodulation inhibition (Nutman, 1949, 1952, 1962). Autoregulation is also prevalent before the establishment of nitrogen-fixing nodules. Pierce and Bauer (1983) showed that an initial inoculation of soybeans several hours prior to a second inoculation, reduced the capacity of the latter to induce nodule formation. Similarly, nodule formation on one half of a soybean root system is inhibited by prior inoculation of the other half,even before the onset of nitrogen fixation (Kosslak and Bohlool, 1984). The results reported here indicate that *nts*382 and *nts*1116 are mutants in the autoregulation pathway. Under nitrate-free conditions, both mutants formed many more nodules than Bragg (Table 6.3,

Figs. 6.2 and 6.3). In addition, these mutants were considerably less sensitive to nitrate inhibition of nodule growth. While Bragg showed a characteristic decrease in nodule fresh weight per plant with the addition of nitrate to the nutrient solution, this trend was not seen in *nts*382 (Table 6.3) or *nts*1116 (Figure 6.2). Therefore, it is likely that autoregulation and nitrate act on nodule growth via a common regulatory pathway.

As a consequence of a larger nodule mass, nitrate-grown *nts* mutants had higher nitrogenase (acetylene reduction) activity per plant that did wild-type controls. Depending on the selected line and the age at harvest, *nts* mutants had seven to nine times the acetylene reduction activity of the wild-type (Table 6.4). In the absence of nitrate, *nts*382 had the same activity as wild-type plants. This result reflected decreased specific nitrogenase activity (activity per unit of nodule mass) in the mutant (Fig. 6.4), since *nts*382 had a larger nodule mass. Clearly, the control of nodule initiation and nodule growth is separate from nodule functioning in *nts*382, and these prolifically-nodulating plants cannot support optimum levels of specific nitrogenase activity.

Specific nitrogenase activity by wild-type soybeans was lower in nitrate-grown plants than in N_2-dependent plants (Fig. 6.4). This was not the case in *nts*382 plants. In this mutant, nitrate treatment did not further depress specific nitrogenase activity. Specific nitrogenase activity in *nts*382 plants from both growth conditions approximated specific activity in nitrate-inhibited wild-type plants. However, it has not yet been established whether the factor(s) limiting nodule function is the same in *nts*382 nodules and nitrate-inhibited wild-type nodules.

An additional characteristic of *nts* mutants was that they were generally smaller than wild-type siblings or parent cultivar Bragg. This is illustrated for *nts*382 in Table 6.3 and Figure 6.3. Perhaps prolific nodule development decreased the assimilates available to other parts of the plant. However, there appeared to be variation between mutants in both the degree of nodulation and plant growth, such that an inverse relationship was evident between these two parameters. Furthermore, some marginal *nts* mutants (not described here) showed up to 40% increased leaf area compared to the wild-type, while still exhibiting a marginal *nts* phenotype. This genetic diversity may modulate the correlation between reduced growth and supernodulation and aid the agronomic application of this mutant phenotype.

Mutant *nts*382 is very similar to a pea mutant designated nod_3 that has enhanced nodulation in the presence of nitrate (Jacobsen, 1984; Jacobsen and Feenstra, 1984). Like *nts*382, nod_3 nodulates prolifically in the absence of nitrate (Jacobsen and Feenstra, 1984) and also has the ability to utilize nitrate (Jacobsen, 1984). A more-detailed description of the nodulation characteristics of *nts*382 is presented in Chapter 7.

CHAPTER SEVEN

A SUPERNODULATION AND NITRATE-TOLERANT SYMBIOTIC (*nts*) SOYBEAN MUTANT

7.1 Introduction

Nodule formation in legumes is tightly regulated. Indeed, symbiotic development is subject to both external factors and internal (or 'autoregulation') control mechanisms. Several environmental conditions, such as light intensity (photosynthate- or non-photosynthate-mediated), temperature, pH and soil moisture, influence nodulation (Lie, 1974). However, under optimum conditions for plant growth, exogenous nitrate represents a major environmental factor controlling the extent of symbiosis (Harper, 1976; Herridge, 1982; Carroll and Gresshoff, 1983). Small amounts of nitrate have been demonstrated to stimulate nodulation. Above these minute concentrations, however, nodule fresh weight is inversely related to the level of nitrate in the growth medium (e.g. Fig. 6.1 in Chapter 6). To a lesser extent, other sources of combined nitrogen have also been shown to inhibit nodulation (Richardson *et al.*, 1957; van Schreven, 1959; Gibson and Nutman, 1960; Darbyshire, 1966; Dart and Wildon, 1970). The extent of this inhibition does vary considerably with the legume species, the form of combined nitrogen administered and the experimental system. Some sources of combined nitrogen, for example urea or NH_4^+, cause acidification of the growth medium (Israel and Jackson, 1982) and the inhibitory effects of these nitrogen sources may be mediated indirectly through a reduction in pH, rather than through the nitrogen status of the plant. To circumvent this complication, Vigue *et al.* (1977) controlled pH fluctuations by the inclusion of a pH-buffering carboxy resin in the pots (Lahav *et al.*, 1976). In this system using soybeans, nitrate but not urea suppressed nodule fresh weight per plant at the range of concentrations tested. This was partially explained by reduced uptake of nitrogen in urea-fed plants. In contrast to

the effect of nitrate and urea on nodule mass, rates of acetylene reduction per unit of nodule mass were similar for nitrate and urea treatments (Vigue *et al.*, 1977).

In the absence of externally supplied combined nitrogen, nodulation is tightly regulated with the number of infections greatly exceeding the final number of mature nodules (Bauer, 1981). Interruption of invasion is related to the effectiveness of the host-*Rhizobium* association (Nutman, 1949) as well as to other internal factors not directly related to the nitrogen status of the plant (Pierce and Bauer, 1983). Generally, ineffective strains of *Rhizobium* form more nodules, especially after the initial stages of nodule formation (Nutman, 1949). Nutman (1952) also showed that excision of effective (but not ineffective) red clover nodules resulted in a transient increase in the number of nodules subsequently formed. In fact, removal of the nodule meristem of effective nodules was sufficient to stimulate subsequent nodule development. The effect of nodule meristem excision on nitrogenase activity was not considered, but the argument that an inhibitory factor emanating from the growing point, and not the bacterial tissue, of the nodule was supported by the finding that excision of the main root tip had the same effect as nodule meristem removal. Clear evidence for internal regulation or autoregulation independent of nitrogen fixation and in the early stages of nodule initiation was reported by Pierce and Bauer (1983). Using the spot inoculation technique (Turgeon and Bauer, 1983), they showed that inoculation of soybean roots with *R. japonicum* several hours prior to a second inoculation substantially reduced nodulation by the second inoculation. Studies by Calvert *et al.* (1984b) characterized this rapid regulatory phenomenon (Pierce

and Bauer, 1983) further and showed that suppression of nodulation due to prior inoculation is mediated through suppression of nodule emergence rather than by inhibition of root hair infection. In a split-root system for soybeans, Kosslak and Bohlool (1984) showed that autoregulation prior to nodule appearance and nitrogenase activity was not restricted to root tissue immediately adjacent to the inoculated area, since prior inoculation of one side of a split-root suppressed nodulation on the other side (Kosslak and Bohlool, 1984). Clearly, the pending fruition of an infection is subject to internal (or auto-) regulation that exists both prior and subsequent to nitrogen fixation.

In the previous chapter, the isolation of 15 independent soybean mutants that continued to nodulate in the presence of nitrate was described. These lines were designated *nts* (*n*itrate-*t*olerant *s*ymbiosis) mutants. In this chapter, the nodulation characteristics of mutant line *nts*382 are described in greater detail. The effects of various nitrogen sources on nodulation, nitrogen fixation and plant growth are described, as well as the influence of two *Rhizobium japonicum* strains on the expression of the supernodulation phenotype.

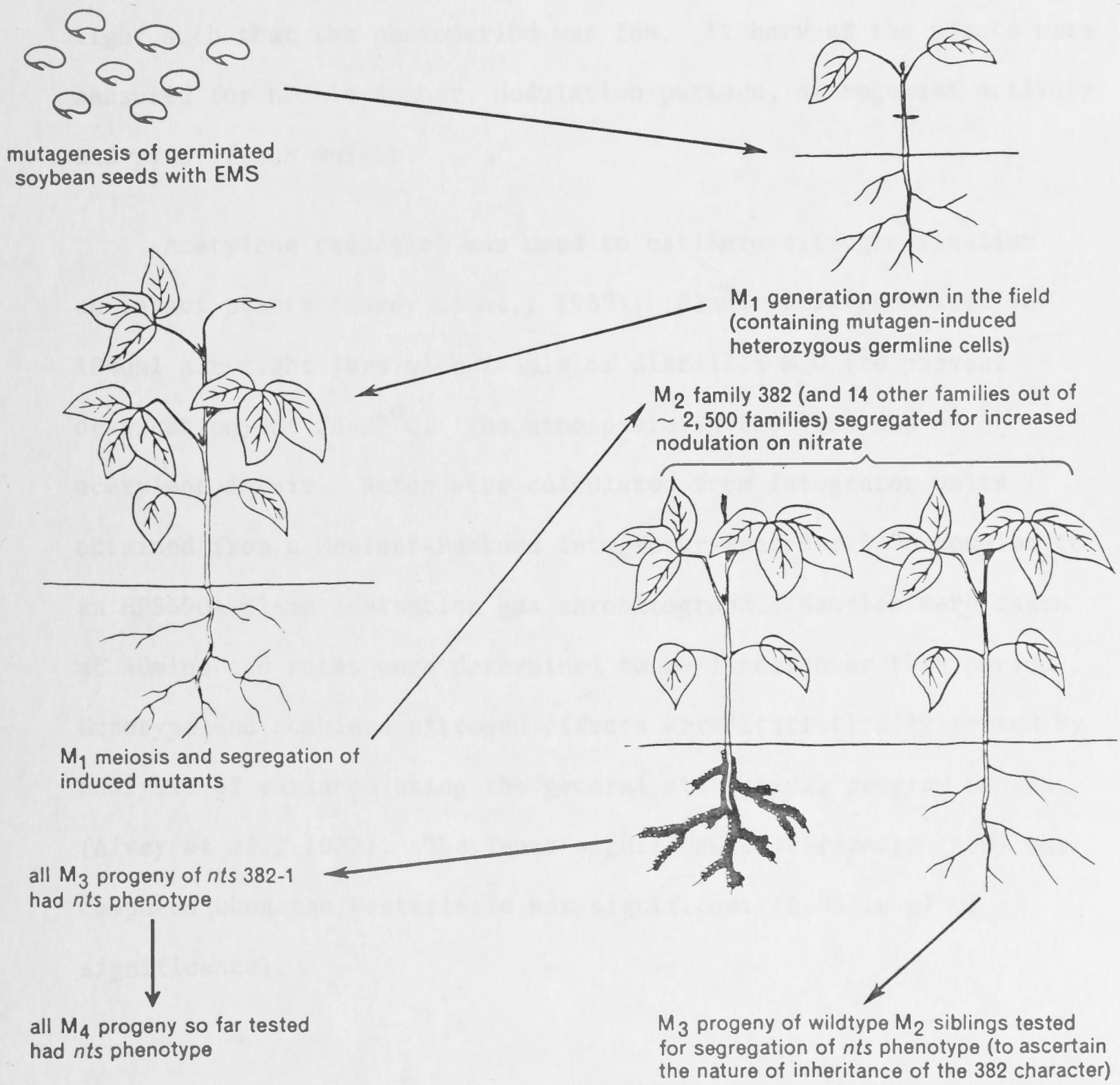
7.2 Materials and Methods

Soybean (*Glycine max* (L.) Merr.) cultivar Bragg was used in this study. Mutant line *nts*382 was selected for increased nodulation in the presence of high nitrate concentrations. The isolation procedure has been described in detail (Chapter 6) and is summarized in Figure 7.1. M_4 plants of mutant line *nts*382 were used here. Plants were cultured in either pots of river sand or in Leonard jars (Gibson, 1980).

In experiments testing the effect of various nitrogen sources on nodulation and N_2 (C_2H_2) fixation, plants were cultured in 20cm diam. pots of sand. Three Bragg or *nts*382 seeds were planted 1cm below the surface and inoculated with *Rhizobium japonicum* strain CB1809 (= USDA136) or USDA110 . The pots were re-inoculated at day 4. The nutrient solution was as used by Herridge (1977), except that all nutrients other than $CaCl_2$ and the nitrogen source were administered at quarter strength for the first 2 weeks. KNO_3 (2.75 and 5.5mM), urea (5.5mM, i.e. 11mM N), NH_4Cl (5.5mM), NH_4NO_3 (5.5mM, i.e. 11mM N) and KCl (as control) were added to the nutrient solution as required. The pots were watered daily with 700mls of nutrient solution, which was sufficient to flush out residual nutrients from the previous watering.

To control access of *R. japonicum* strains to the roots (Gibson, 1980), Bragg and *nts*382 were cultured in Leonard jars. Seeds were surface-sterilized by rinsing in 95% ethanol followed by immersion of the seeds for 10mins in 3% sodium hypochlorite. After several rinses in sterile distilled water, the seeds were transferred to water agar and germinated at 28°C in the dark. When the radical was approximately 0.5 - 1cm long (2-3 days after sterilization) the

Figure 7.1: Schematic representation of the isolation of *nts*382 from mutagenized Bragg soybeans.


mutagenesis of germinated soybean seeds with EMS
M1 generation grown in the field (containing mutagen-induced heterozygous germline cells)
M2 family 382 (and 14 other families out of ~ 2,500 families) segregated for increased nodulation on nitrate
M1 meiosis and segregation of induced mutants
all M3 progeny of nts 382-1 had nts phenotype
all M4 progeny so far tested had nts phenotype
M3 progeny of wildtype M2 siblings tested for segregation of nts phenotype (to ascertain the nature of inheritance of the 382 character)

seedlings were transferred to Leonard jars (N-free or supplemented with KNO_3 or urea). The jars either remained uninoculated or were inoculated with *R. japonicum* strains CB1809 or USDA110.

The plants were cultured in a temperature-controlled glasshouse (mean maximum temperature = 26.9°C; mean minimum temperature = 14.8°C) at a latitude of 37° 17' S. Incandescent bulbs supplemented natural light such that the photoperiod was 16h. At harvest the plants were measured for nodule number, nodulation pattern, nitrogenase activity and plant fresh weight.

Acetylene reduction was used to estimate nitrogen fixation on intact plants (Hardy *et al.*, 1968). Plants were incubated in 1040ml air-tight jars with 2-3mls of distilled H_2O (to prevent dessication) at 25-27°C. The atmosphere in the jars was 6% acetylene in air. Rates were calculated from integrator units obtained from a Hewlett-Packard Integrator-Recorder 3390 coupled to an HP5590A flame ionisation gas chromatograph. Samples were taken at 40mins and rates were determined to be linear over that period. Genotype and combined nitrogen effects were statistically tested by analysis of variance using the general statistical program Genstat (Alvey *et al.*, 1977). The least significance difference (LSD) was computed when the F-statistic was significant (0.05 level of significance).

7.3 Results

7.3.1 Effect of combined nitrogen on nodulation, nitrogenase activity and growth.

Bragg and *nts*382 were inoculated with *R. japonicum* CB1809 and cultured for 4 weeks in the presence or absence of various combined nitrogen sources. The nitrogen-free treatment received 5.5mM KCl as a control.

7.3.1.1 Nodule number. Under all the conditions tested, four week-old *nts*382 plants had considerably more nodules than wild-type Bragg plants (Fig. 7.2). In the parent cultivar, all nitrogen sources reduced nodule number (Figs. 7.2 and 7.3). In contrast, mutant line *nts*382 grown on KNO_3 or urea had increased nodule number per plant over that of the KCl controls (Fig. 7.2). When data for nodule number are expressed per plant fresh weight, nodule number in *nts*382 was unaffected by increasing KNO_3 concentration (Fig. 7.3). Urea (5.5mM), on the other hand, caused a small reduction in nodule number per plant biomass in the mutant. The relative degree of inhibition of nodule formation by urea was greater in Bragg than in *nts*382 (Fig. 7.3). Mutant line *nts*382 formed nodules on 5.5mM NH_4Cl and NH_4NO_3, which totally prevented nodule formation in Bragg (Figs. 7.2 and 7.3). Ammonium chloride inhibited growth to a larger extent than did ammonium nitrate (Table 7.1), however NH_4NO_3 was more inhibitory than NH_4Cl on nodule formation in *nts*382 (Fig. 7.3).

7.3.1.2 Nitrogenase (acetylene reduction) activity per plant biomass. In Bragg, supplementing the nutrient media with nitrate or urea caused a substantial reduction in nitrogenase activity measurable 4 weeks after planting (Fig. 7.4). In contrast, 2.75mM KNO_3 significantly stimulated acetylene reduction per gram plant fresh weight in *nts*382.

Figure 7.2: Effect of nitrogen source on nodule number per plant in Bragg and *nts*382 plants. Plants were cultured and harvested as in Table 1. Raw data (panel A) required $\log_e$ transformation (panel B) to satisfy assumptions for an analysis of variance. Each column represents the mean of 4 to 8 plants. Genotype, combined nitrogen and interaction effects were significant.

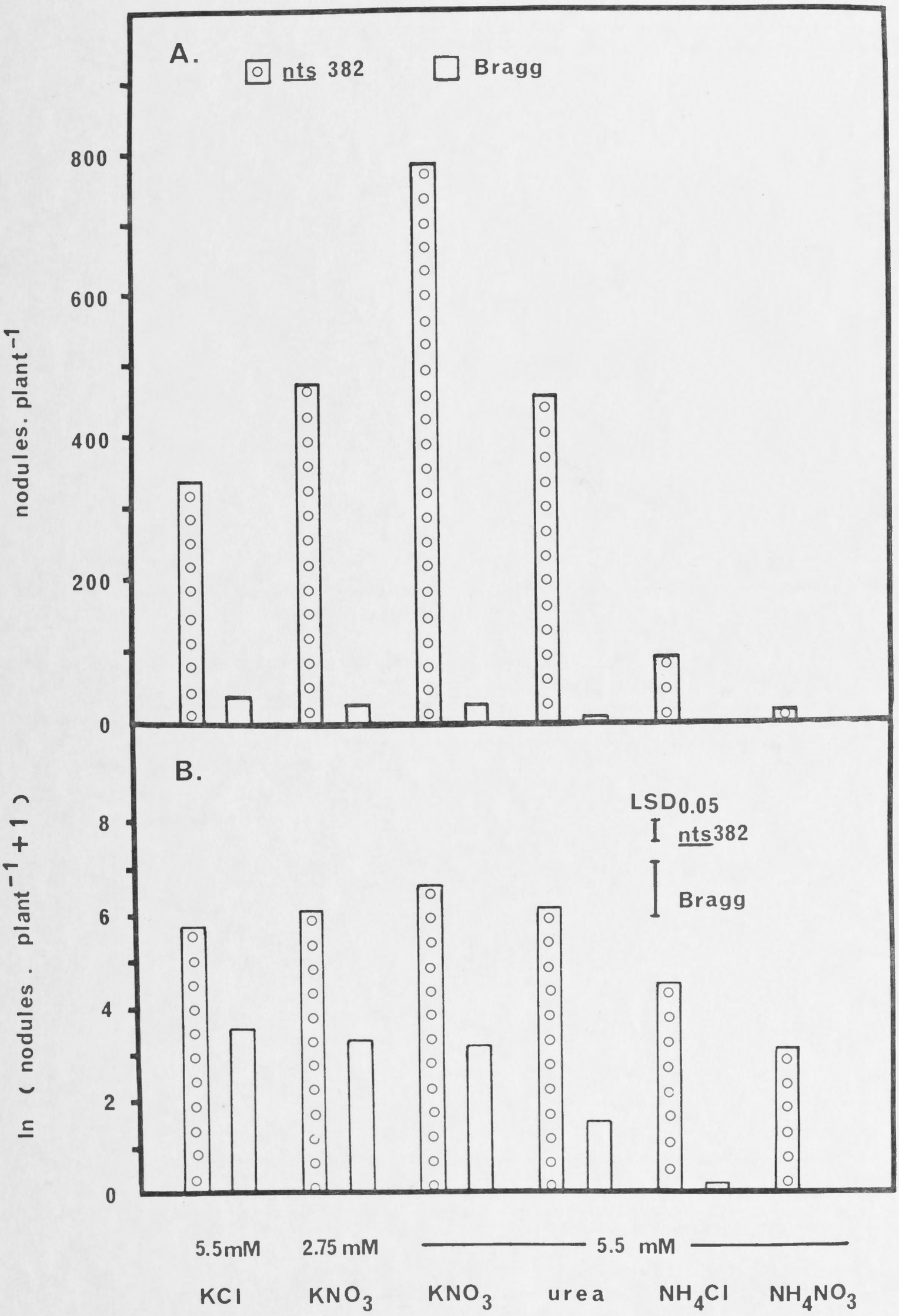

A.
nts 382
Bragg
800
600
400
200
0
nodules. plant^{-1}
B.
LSD$_{0.05}$
nts382
Bragg
8
6
4
2
0
ln (nodules . plant^{-1} + 1)
5.5mM
2.75 mM
5.5 mM
KCl
KNO$_3$
KNO$_3$
urea
NH$_4$Cl
NH$_4$NO$_3$

Figure 7.3: Effect of nitrogen source on nodule number per plant biomass in Bragg and *nts*382. Plants were cultured and harvested as in Table 1. Raw data (panel A) required $\log_e$ transformation (panel B) to satisfy assumptions for an analysis of variance. Each column represents the mean of 4 to 8 plants. Genotype, combined nitrogen and interaction effects were significant.

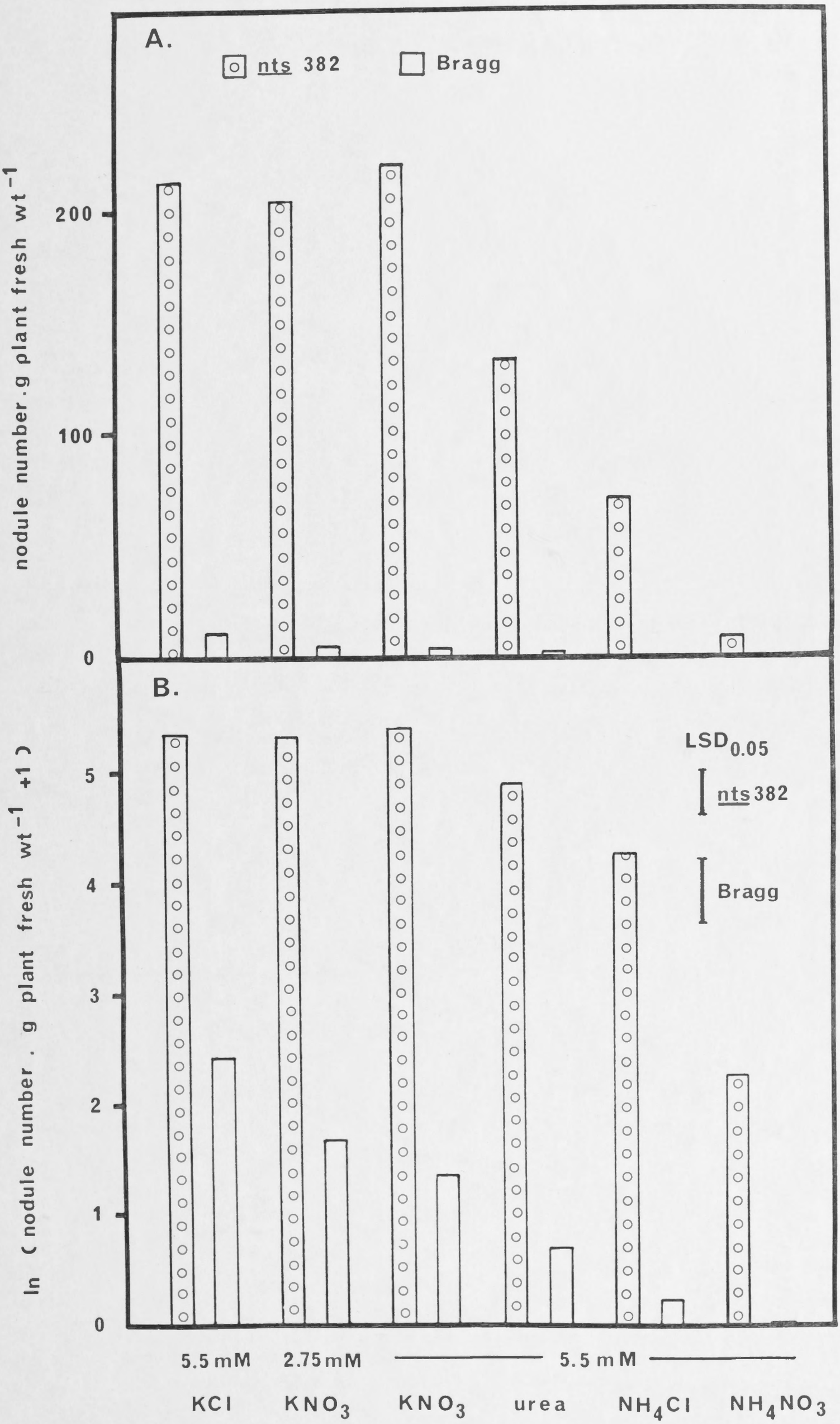

A.
nts 382
Bragg
nodule number.g plant fresh wt^{-1}
200
100
0
B.
ln (nodule number . g plant fresh wt^{-1} +1)
5
4
3
2
1
0
LSD$_{0.05}$
nts 382
Bragg
5.5 mM
2.75 mM
5.5 mM
KCl
KNO$_3$
KNO$_3$
urea
NH$_4$Cl
NH$_4$NO$_3$

Table 7.1: Plant fresh weight of Bragg and *nts*382 plants cultured for 4 weeks on various nitrogen sources. The plants were grown in 20cm sand pots as described in Materials and Methods.

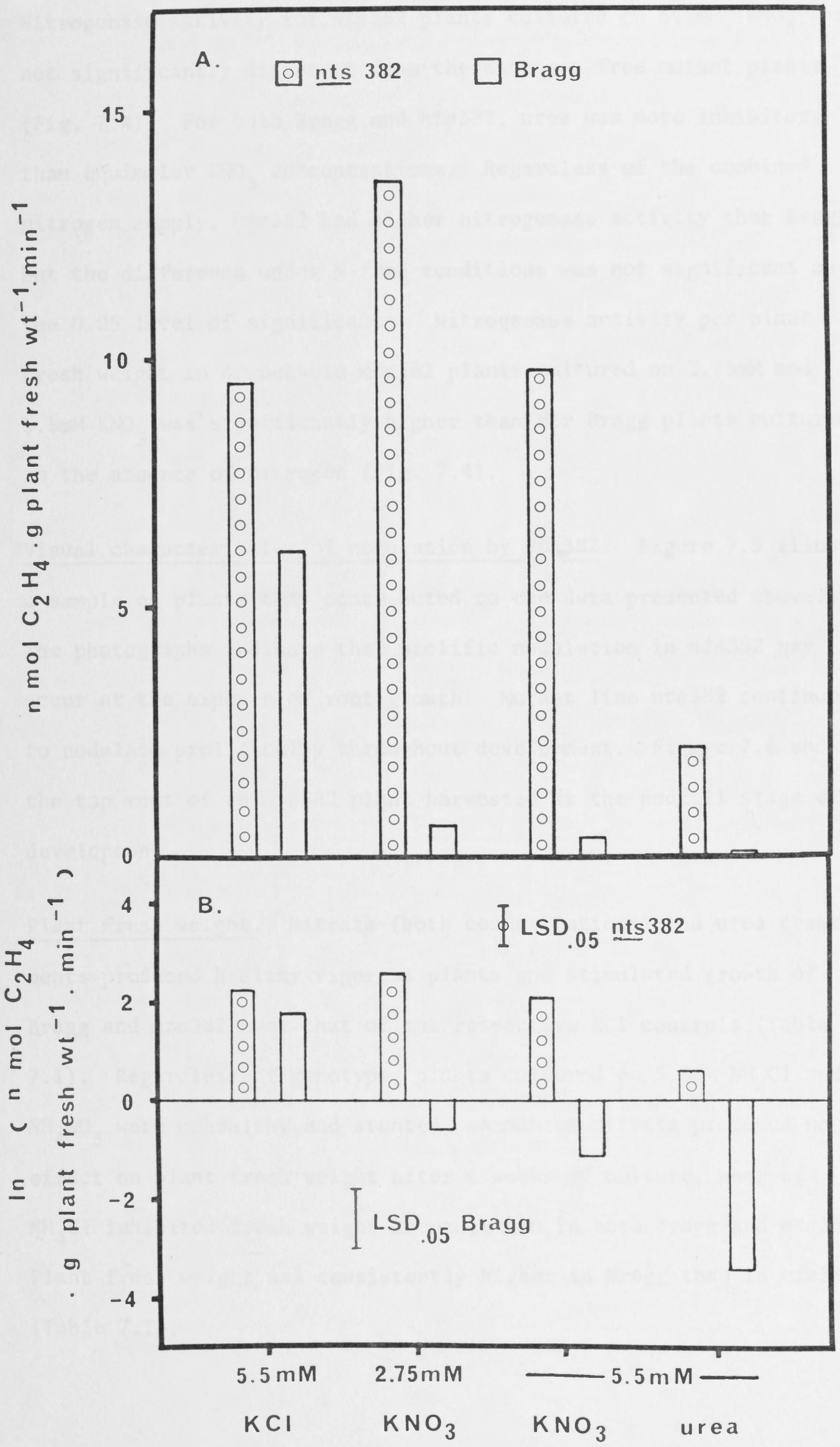
Table 7.1

Nitrogen Source	Plant fresh weight (g)[a]	
	Bragg	*nts*382
0mM (5.5mM KCl)	3.24 (100)[b]	1.96 (100)[b]
2.75mM KNO_3	5.61 (173)	2.37 (121)
5.5mM KNO_3	8.05 (248)	3.68 (188)
5.5mM urea	4.80 (148)	3.51 (179)
5.5mM NH_4Cl	2.12 (65)	1.27 (65)
5.5mM NH_4NO_3	3.04 (94)	2.18 (111)

[a] $LSD_{0.05}$ = 1.56 for Bragg and 1.08 for *nts*382

[b] plant fresh weight as % of 5.5mM KCl control.

Figure 7.4: Effect of potassium nitrate and urea on nitrogenase (acetylene reduction) activity in Bragg and *nts*382 plants. Plants were cultured and harvested as in Table 1. Raw data (panel A) required $\log_e$ transformation (panel B) to satisfy assumptions for an analysis of variance. Each column represents the mean of 4 to 8 plants. Genotype, combined nitrogen and interaction effects were significant.


A.
nts 382
Bragg
15
10
5
0
n mol C_2H_4 .g plant fresh wt^{-1}.min^{-1}
B.
$LSD_{.05}$ nts382
$LSD_{.05}$ Bragg
4
2
0
-2
-4
ln (n mol C_2H_4 . g plant fresh wt^{-1}. min^{-1})
5.5mM
KCl
2.75mM
KNO_3
5.5mM
KNO_3
urea

Nitrogenase activity for *nts*382 plants cultured on 5.5mM KNO_3 was not significantly different from the nitrogen-free mutant plants (Fig. 7.4). For both Bragg and *nts*382, urea was more inhibitory than equimolar KNO_3 concentrations. Regardless of the combined nitrogen supply, *nts*382 had higher nitrogenase activity than Bragg, but the difference under N-free conditions was not significant at the 0.05 level of significance. Nitrogenase activity per plant fresh weight in 4 week-old *nts*382 plants cultured on 2.75mM and 5.5mM KNO_3 was significantly higher than for Bragg plants cultured in the absence of nitrogen (Fig. 7.4).

7.3.1.3 Visual characteristics of nodulation by *nts*382. Figure 7.5 illustrates a sample of plants that contributed to the data presented above. The photographs indicate that prolific nodulation in *nts*382 may occur at the expense of root growth. Mutant line *nts*382 continued to nodulate prolifically throughout development. Figure 7.6 shows the tap root of an *nts*382 plant harvested at the podfill stage of development.

7.3.1.4 Plant fresh weight. Nitrate (both concentrations) and urea treatments produced healthy vigorous plants and stimulated growth of Bragg and *nts*382 over that of the respective KCl controls (Table 7.1). Regardless of genotype, plants cultured on 5.5mM NH_4Cl and NH_4NO_3 were unhealthy and stunted. Ammonium nitrate produced no effect on plant fresh weight after 4 weeks of culture, whereas NH_4Cl inhibited fresh weight accumulation in both Bragg and *nts*382. Plant fresh weight was consistently higher in Bragg than in *nts*382 (Table 7.1).

Figure 7.5: Effect of potassium nitrate and urea on nodulation in Bragg (A) and *nts*382 (B) plants. Plants were cultured and harvested as in Table 1.

A


Bragg
5.5 mM KCl
5.5 mM urea
2.75 mM KNO_3
5.5 mM KNO_3

B


nts 382
5.5 mM KCl
5.5 mM urea
2.75 mM KNO_3
5.5 mM KNO_3

Figure 7.6: Nodulation of *nts*382 at the podfill stage of development. A) the tap root of an *nts*382 plant that had been cultured in a 25cm pot of vermiculite. This plant had received slow-release nitrogen fertilizer throughout growth. B) close-up of Figure 7.6A .

A

B

7.3.2 Axenic culture in Leonard jars. The observations obtained from pots inoculated with *R. japonicum* strain CB1809 and described above were confirmed in Leonard jars supplemented with KNO_3 and urea. Both *R. japonicum* stains tested, namely CB1809 and USDA110, elicited the *nts* phenotype in mutant line 382. Uninoculated controls of *nts*382 and Bragg did not nodulate.

7.3.3 Nodulation pattern. Table 7.2 shows the tap root nodulation pattern of N_2-dependent *nts*382 and Bragg plants. Tap root length was less in nodulated *nts*382 plants than in Bragg plants cultured under identical conditions (Table 7.2). The tap root nodulation interval is defined as the distance between the uppermost and lowermost nodule on the tap root. This parameter was larger in *nts*382, both in absolute terms (two times that of Bragg) and when expressed as a percentage of tap root length (three times that of Bragg). Nodule density, expressed on the total tap root length was nine times higher in *nts*382 than in Bragg. Within the nodulation interval, the nodule density was two and one half times higher in the mutant line (Table 7.2). A similar contrast in nodulation pattern was observed on lateral roots and, furthermore, on *nts*382 plants cultured on various nitrogen sources (Fig. 7.5).

Table 7.2: Tap-root nodulation pattern of N_2-dependent *nts*382 and Bragg plants. Plants were inoculated with *R. japonicum* USDA110 and harvested 9 weeks after planting. The pots were watered daily with N-free nutrient solution throughout the experiment.

Table 7.2

Tap root parameter	Soybean line		
	*nts*382[a]	Bragg[b]	$LSD_{0.05}$
Root length (cm)	16.6	28.1	3.1
Nodulation interval (cm)	14.4	7.6	2.1
Nodulation interval (% of root length)	86.5	27.1	6.7
Nodule density on root length ($nodules.cm^{-1}$)	4.32 (2.0)[c]	0.47 (0.67)	-(0.15)
Nodule density on nodulation interval ($nodules.cm^{-1}$)	5.07	2.10	0.99

[a] each entry in the Table for *nts*382 represents the mean of 7 plants

[b] each entry in the Table for Bragg represents the mean of 28 plants

[c] raw data required square-root transformation to satisfy assumptions for an analysis of variance; means and LSD of transformed data are shown in brackets.

7.4 Discussion

Mutant line *nts*382 was one of several *nts* (nitrate-tolerant symbiosis) mutants selected from an M_2 population of parent cultivar Bragg for increased nodulation in the presence of nitrate (Chapter 6). Individual M_2 families (resulting from a single mutagenized M_1 seed) were originally screened. M_2 family 382 segregated for the *nts* character (Fig. 7.1), as did all of the M_2 *nts* mutant families. This observation indicated that each of the mutant families arose from independent mutation events and that the mutants were a result of the mutagenesis program (Chapter 6). M_2 *nts*382 selections were homozygous for the mutation, since all M_3 progeny derived thereof had the *nts* phenotype, as did all M_4 progeny, including the plants used in some experiments described here. In Chapter 8 that follows, data are presented that indicate that the mutant character in *nts*382 is inherited as a Mendelian recessive.

Regardless of the presence or absence of combined nitrogen, *nts*382 nodulated more than the parent cultivar Bragg. This trend was consistent over a range of combined nitrogen sources that caused varying degrees of inhibition of nodulation in Bragg. For example, under conducive conditions for nodulation, 4 week-old N_2-dependent *nts*382 plants had 9 times the nodule number of N_2-dependent Bragg plants. Similarly under conditions that totally prevented nodulation in Bragg (5.5mM NH_4Cl or NH_4NO_3), *nts*382 plants were still nodulated (Figs. 7.2 and 7.3). The mutant line also had increased nodulation in Leonard jars, in deep soil pots (soil obtained from a soybean field at Breeza, NSW, Australia), and in Georgian (USA) fieldplots (J. Key, *pers. comm.*). Nodule initiation and nodule growth are co-ordinated in *nts*382, and *nts*382 plants have a considerably

larger nodule mass than wild-type plants (Chapters 6 and 8). The term supernodulator is used to describe *nts*382, since this soybean genotype has an increased nodule mass under a wide range of environmental conditions.

Although *nts*382 is a supernodulator, it is not a constitutive nodulator, since it still requires the inducer (i.e. *R. japonicum*) to be present. The two strains of *R. japonicum* used, namely CB1809 and USDA110, elicited the *nts* phenotype in the mutant line. Both these strains form an effective symbiosis with the parent cultivar. A wider spectrum of fast- and slow-growing *R. japonicum* strains, that vary in their ability to nodulate the parent cultivar, were tested on *nts*382 to ascertain whether the *nts* character confers a change in the promiscuity of the host plant. Those strains that nodulated Bragg elicited the mutant phenotype on *nts*382, and strains that were nod^- on the parent cultivar were also unable to induce nodule formation on *nts*382 (E. Appelbaum, *pers. comm.*).

In Bragg, all nitrogen sources caused a significant reduction in the symbiotic parameters that were measured (Figs. 7.2, 7.3, 7.4 and 7.5). In contrast, enriching the nutrient solution with KNO_3 did not inhibit any symbiotic parameters in *nts*382 and was in some cases stimulatory (Figs. 7.2 and 7.4). Urea marginally stimulated nodule number per plant but significantly inhibited nodule number and nitrogenase activity per gram plant fresh weight in the mutant line. Consistent with the comparative effects of KNO_3 and urea on *nts*382, urea was also more severe on symbiotic development in Bragg (Figs. 7.2, 7.3 and 7.4). This is different to what Vigue *et al.* (1977) found with soybean cultivar Steele. Using a carboxy resin to buffer the culture media (Lahav *et al.*, 1976), they demonstrated that

nitrate was more inhibitory on nodulation than was urea. To minimize the drop in pH associated with urea and ammonium utilization (Israel and Jackson, 1982), the pots in our experiments were flushed daily with 700mls of nutrient solution. This procedure, without a specific buffering agent, produced healthy vigorous urea-fed plants that had significantly stimulated fresh weights in Bragg and *nts*382 (Table 7.1). Nevertheless, it is unclear whether pH fluctuations played a role in inhibition of the symbiosis by urea, NH_4Cl and NH_4NO_3. This however is not a contentious issue, since the salient feature of *nts*382 illustrated by these experiments is that it consistently nodulated prolifically in comparison to parent cultivar Bragg, regardless of the environmental conditions imposed by the provision of various nitrogen sources.

Reiterating a conclusion from Chapter 6, the results presented in this chapter showed that prolific nodulation by *nts*382 was not confined to culture on nitrate, and it is therefore unlikely that increased nodulation on nitrate resulted from an inability to utilize nitrate. KNO_3 enhanced the growth of *nts*382 plants (Table 7.1) and, furthermore, *nts*382 had the same nitrate reductase activity as Bragg (Chapter 6). Indeed, *nts*382 is a mutant in the regulation of nodule initiation and nodule development, and the autoregulation mechanism normally limiting nodulation in wild-type soybeans (Pierce and Bauer, 1983; Calvert *et al.*, 1984b) is anomalous. The nodulation interval is extended and nodule density is increased in *nts*382 (Table 7.2, Fig. 7.5). Although nodule emergence and subsequent nodule growth are coordinately controlled in *nts*382, specific nitrogenase activity (activity per gram nodule fresh weight) is not coordinately regulated with these two nodule development parameters in the mutant (Chapter 6). The fact that *nts*382 plants have increased nitrogenase activity per plant biomass under adverse conditions for symbiotic development is a result of increased nodule mass.

In *nts*382 mutant plants regulation of nodulation by environmental conditions (such as by nitrate supply) is coordinated with internal (or auto-) regulation. The supernodulation observed in *nts*382 under N-free conditions persisted when combined nitrogen was added to the nutrient media. The increased tolerance of *nts*382 to nitrate and other combined nitrogen sources was seen in an accentuation in the magnitude of difference between *nts*382 and Bragg as nitrogen sources were added to the nutrient solution (Table 7.3). For example, in the absence of combined nitrogen, *nts*382 plants had 9 times as many nodules as Bragg plants (Table 7.3). As combined nitrogen was added to the nutrient solution the degree of inhibition in Bragg was not seen in *nts*382 and for plants cultured on 5.5mM urea, for example, *nts*382 had 65 times as many nodules per plant as did Bragg. A similar trend was evident for all the other symbiotic parameters measured (Table 7.3). Clearly, *nts*382 is a mutant in the autoregulation pathway and is also less sensitive to regulation by external conditions. Pea mutant nod_3 (Jacobsen and Feenstra, 1984) is similar to *nts*382 in that it nodulated more than the parent cultivar in the absence of nitrate and also nodulation was less affected by nitrate in nod_3.

In addition to being useful in studying regulation of nodule development in general, *nts*382 is of immense value for critically analysing and refining some hypotheses that have been put forward to explain nitrate inhibition of nodule development. The carbohydrate deprivation hypothesis argues that nitrate reduction and assimilation deprives the nodule of carbohydrate necessary for growth and development. The alternative hypothesis that has also received support is that products on nitrate reduction, particularly nitrite, are responsible for nitrate inhibition. The nodulation

Table 7.3: Symbiotic parameters of *nts*382 expressed as a proportion of Bragg. Each datum in the Table was computed by dividing the value of the symbiotic parameter for *nts*382 by the value for Bragg cultured under identical conditions (i.e. *nts*382 ÷ Bragg). Nitrogen sources are listed in order of increasing severity on nodulation in Bragg. Plants were harvested 4 weeks after planting. The inoculant strain was *R. japonicum* CB1809.

Table 7.3

Nitrogen Source	Symbiotic parameters[a]		
	nodules per plant[b]	nodules per g plant fresh weight[c]	nitrogenase activity[d]
0mM (5.5mM KCl)	9	21	1.6
2.75mM KNO_3	18	51	22.6
5.5mM KNO_3	31	73	29.5
5.5mM urea	65	132	36.0
5.5mM NH_4Cl[e]	∞	∞	-
5.5mM NH_4NO_3[e]	∞	∞	-

[a] *nts*382 ÷ Bragg

[b,c,d] data from Figure 2, Figure 3 and Figure 4, respectively

[e] NH_4Cl and NH_4NO_3 totally prevented nodule formation in Bragg.

characteristics of *nts*382 and Bragg indicate that mass flow carbohydrate deprivation or products of nitrate reduction cannot directly cause inhibition of nodule development. Growth of *nts*382 was stimulated by potassium nitrate (Table 7.1) and *nts*382 had normal nitrate reductase activity (Chapter 6) indicating the mutant did utilize nitrate and yet nodule development was not inhibited early in plant development (0 to 4 weeks after planting). Carbohydrate deprivation or products of nitrate reduction may inhibit nodule development in wild-type but it must be mediated through the control mechanism that is anomalous in *nts*382.

CHAPTER EIGHT

INHERITANCE OF THE MUTANT CHARACTER IN *nts*382, *nts*1007, *nts*183 AND *nts*733.

8.1 Introduction

Nodulation mutants have been reported in several legume species (see Chapter 1). Generally, where the nature of inheritance is known, the mutations are simple recessives or dominants. However, two variations from simple recessive or dominant inheritance have been reported in pea (*Pisum sativum*). A temperature-dependent dominant gene conditioning non-nodulation has been identified (Lie, 1971; Lie *et al.*, 1976). This mutation is strain-specific (Lie, 1971) and the gene has been designated *Sym*1 (Holl and La Rue, 1976). Also in pea, incomplete dominance for a non-nodulation trait has been noted in two Afghanistan lines (Ohlendorf, 1983a, 1983b). These two lines, homozygous for gene A and gene B respectively, exhibit strain-specific non-nodulation (Ohlendorf, 1983a). A spectrum of intermediate nodulation phenotypes occurred in F_1 plants derived from a cross between a pure-breeding nodulating line and a pure-breeding line carrying either gene A of gene B (Ohlendorf, 1983b). Host factor *r* conditions non-nodulation in red clover and it is also exceptional in that it is associated with a maternally transmitted component (Nutman, 1981).

Prior to this study (Chapters 6, 7 and 10), there were only four nodulation mutants reported in soybean. These naturally-derived variations are inherited as simple recessive or dominant mutations. Plants homozygous for the recessive gene rj_1 generally do not nodulate (the blockage is partly circumvented by high dose inoculation (La Favre and Eaglesham, 1984)). In addition to rj_1, three dominant genes affecting nitrogen fixation have been reported in soybean. Rj_2 (Caldwell, 1966), Rj_3 (Vest, 1970) and Rj_4 (Vest and Caldwell, 1972) condition strain-specific ineffective nodulation.

Traditionally, the nature of inheritance of characters is ascertained from F_1 progeny and segregation ratios of F_2 progeny derived from crosses between pure-breeding mutant and wild-type lines. In this chapter, an alternative approach is described. M_2 families (derived from mutagenized M_1 seed) segregated for the *nts* phenotype and contained both *nts* mutants and wild-type siblings. If the *nts* character is inherited as a Mendelian recessive, M_2 plants heterozygous for the mutated gene would express the wild-type phenotype. However, M_3 progeny derived from self-fertilization of such plants would segregate 1 mutant to 3 wild-type. In analogy to traditional crossing methods, M_2 heterozygous plants and their resultant M_3 progeny are equivalent to F_1 and F_2 generations, respectively.

Using this approach, M_3 progeny derived from M_2 mutant and wild-type siblings were screened for segregation of the *nts* character. Data are presented in this chapter that illustrate simple recessive inheritance of the mutant character in *nts*382, *nts*1007 and *nts*183. In contrast, the *nts*733 character appears to be incompletely dominant.

8.2 Materials and Methods

M_2 families 382, 1007 and 183 were three of 15 families that segregated for the *nts* phenotype (Chapter 6). Both *nts* variants and wild-type (non-*nts*) siblings were saved to produce M_3 families (i.e. families derived from single M_2 plants). Stability of the mutant character was demonstrated in M_3 progeny of M_2 mutant selections, and those M_3 families derived from wild-type M_2 plants were screened for segregation of the *nts* character (also see Fig. 7.1). Seeds were planted in 25cm diameter pots of river sand (12 seeds per pot). The pots were placed in a temperature-controlled glasshouse (mean maximum temperature = 26.9^{o}C; mean minimum temperature = 14.8^{o}C) and were inoculated with peat cultures of *Rhizobium japonicum* strain USDA110 at day 0 and day 4. The glasshouse was at a latitude of 37^{o} 17'S and incandescent bulbs supplemented natural light such that the photoperiod was 16h. The pots were watered 3 times a week with 1.4L of nutrient solution supplemented with 5mM KNO_3 (as described in Chapter 6). Parent cultivar Bragg and wild-type siblings of *nts* mutants were indistinguishable for nodulation in the presence of nitrate, and therefore nitrate-grown Bragg plants were generally not included in experiments. However, as a N_2-dependent control, Bragg plants were cultured as described above, except that these pots received N-free nutrient solution throughout growth. Plants were harvested at 49 days (1007 and Bragg) and 51 days (382 and 183) after planting. Plants were measured for nodule number (on tap root and lateral roots), nodule fresh weight, nitrogenase activity and plant fresh weight. Acetylene reduction was used to estimate nitrogen fixation on intact plants, as described in Chapter 6.

In contrast to families 1007, 382 and 183, M_2 family 733 segregated for three phenotypes: *nts*, marginal *nts* and wild-type. M_3 progeny were screened for the *nts* phenotype as described above, except that *R. japonicum* strain CB1809 (USDA136) was sometimes used as inoculant instead of strain USDA110. The plants were harvested at 59-63 days after planting.

Data were statistically tested by analysis of variance using the general statistical program Genstat (Alvey *et al.*, 1977). The least significant difference (LSD) was computed to compare means only when the F-statistic was significant (0.05 level of significance). Chi-square analysis was used to statistically test segregation ratios. It was necessary to include the Yates correction term in chi-square calculations due to the size of the expected classes (Strickberger, 1976).

8.3 Results

8.3.1 Inheritance of *nts*1007, *nts*382 and *nts*183

M_2 families 1007, 382 and 183 segregated for two nodulation phenotypes: *nts* and wild-type. As shown in Table 8.1, the segregation ratio in M_2 family 1007 was 8 *nts* to 40 wild-type, in family 382, 2 *nts* to 15 wild-type and in family 183, 3 *nts* to 5 wild-type. Mutant *nts* plants in these families were characterized by increased nodulation on nitrate, whereas the wild-type siblings were indistinguishable from parent cultivar Bragg for nodulation on nitrate. For example, seven weeks after planting and culture on 5mM KNO_3, 1007 *nts* plants had 179 $\pm$ 39 ($\pm$ S.D.) nodules, in contrast, 1007 wild-type siblings and Bragg plants had 13 $\pm$ 4 and 19 $\pm$ 7 nodules, respectively. Similarly, 382 and 183 *nts* mutants had 146 $\pm$ 71 and 269 $\pm$ 70 nodules per plant. The respective wild-type siblings in M_2 families 382 and 183 had 26 $\pm$ 11 and 19 $\pm$ 8 nodules per plant.

All M_3 progeny derived from self-fertilized 1007, 382 and 183 mutant plants had increased nodulation in the presence of nitrate. These results indicated that *nts* mutants in these M_2 families were homozygous for the respective mutations.

In contrast, not all M_2 wild-type plants in these families were pure-breeding. In family 1007, three out of 23 wild-type derived M_3 families segregated for the *nts* character (Table 8.1). In selected line 382, one of six M_3 families segregated for the mutant character (Table 8.1). Only one M_2 wild-type in family 183 produced M_3 progeny, and this progeny segregated for the *nts* phenotype (Table 8.1).

Table 8.1: Segregation ratios in M_2 and M_3 progeny of mutant families 1007, 382 and 183.

Table 8.1

Family	Segregation ratio in M_2 family *nts* : wild-type	Frequency of segregating M_3 families[a]	Segregation ratio in M_3 families *nts* : wild-type[b]	Chi-square[c] 1 : 3
1007	8 : 40	$^3/_{23}$	3 : 13	0.17
			12 : 28	0.31
			7 : 24	0.01
382	2 : 15	$^1/_6$	14 : 43	0.00
183	3 : 5	$^1/_1$	8 : 15	0.71

[a] frequency of segregation in M_3 families derived from M_2 wild-type plants; numerator = number of segregating M_3 families, denominator = total number of M_3 families screened.

[b] segregation ratios in individual M_3 families derived from M_2 wild-type plants.

[c] chi-square calculated from the observed segregation ratios and the expected segregation ratio expected for simple Mendelian monogenic recessive inheritance. Chi-square (0.05 level of significance; 1 degree of freedom) = 3.84. Therefore, chi-square values listed in the Table are not significant.

M_3 *nts* segregants were qualitatively similar to the respective M_2 *nts* mutant selections. Furthermore, the mutant to wild-type ratio in segregating M_3 families derived from M_2 wild-types closely approximated 1 mutant to 3 wild-types (Table 8.1). This is the expected segregation ratio for a Mendelian monogenic recessive in progeny derived from a self-fertilized heterozygous plant. Therefore, the *nts*382, *nts*1007 and *nts*183 phenotypes appear to have resulted from monogenic recessive mutations.

Mutant segregants were characterized by substantially increased nodule number, nodule fresh weight (Table 8.2) and nitrogenase activity (Table 8.3) per plant fresh weight. However, specific nitrogenase activity (C_2H_4 produced .g nodule fresh weight^{-1}) was significantly lower in the nitrate-grown mutants than in nitrate-grown wild types (Table 8.3). For comparison of nodulation parameters, N_2-dependent wild-type Bragg plants were harvested with 1007 segregants (49 days after planting). Bragg plants grown without nitrate had 34.0 $\pm$ 9.9 ($\pm$ S.D.) mg nodule fresh weight per g plant fresh weight, compared to 134.6 $\pm$ 47.6 and 4.6 $\pm$ 1.8 for nitrate-grown 1007 *nts* mutants and wild-types, respectively. Acetylene reduction was more efficient in N_2-dependent Bragg plants (342 $\pm$ 7 ($\pm$ S.D.) nmol C_2H_4. g nodule fresh weight^{-1} min^{-1}) than in 1007 *nts* (78 $\pm$ 3) or wild-type (153 $\pm$ 44) segregants that had been cultured on nitrate. However, on a plant biomass basis, nitrogenase (acetylene-reduction) activity was similar in nitrate-grown *nts* segregants and N_2-dependent Bragg plants; 12.74 $\pm$ 2.04 and 10.34 $\pm$ 2.92 nmol C_2H_4 produced. g plant fresh weight^{-1} min^{-1}, respectively. Nitrogenase activity in nitrate-grown 1007 wild-types was significantly lower (Table 8.3).

Table 8.2: Nodulation of *nts* and wild-type siblings in segregating M_3 families of 1007, 382 and 183. The plants were harvested 49 days (1007) and 51 days (382 and 183) after planting and culture on 5mM KNO_3. All seeds were planted on the same day and in the same glasshouse, and were inoculated with *R. japonicum* strain USDA110 at day 0 and day 4. Nodule number and nodule fresh weight are expressed per plant biomass.

Table 8.2

Family	Segregant phenotype		$LSD_{0.05}$
	nts[a]	wild-type[b]	
a) nodule number . g plant fresh wt.$^{-1}$			
1007	67.2 (4.08)[c]	1.5 (0.16)	(0.34)
382	98.9 (9.90)[d]	2.1 (1.42)	(0.42)
183	84.8 (9.19)[d]	4.4 (2.07)	(0.48)
b) mg nodule fresh wt.. g plant fresh wt.$^{-1}$			
1007	134.6 (4.83)[c]	4.6 (1.45)	(0.22)
382	145.3 (12.0)[d]	6.2 (2.5)	(0.5)
183	139.5 (11.8)[d]	11.9 (3.6)	(0.7)

[a] each entry in the Table is the mean of 22, 14 and 8 plants respectively for families 1007, 382 and 183.

[b] each entry in the Table is the mean of 18, 18 and 8 plants respectively for families 1007, 382 and 183.

[c,d] raw data required either $\log_e$ (c) or square-root (d) transformation to satisfy assumptions for an analysis of variance; means and LSD of transformed data are shown in brackets.

Table 8.3: Nitrogenase (acetylene reduction) activity in *nts* and wild-type siblings from segregating M_3 families of 1007, 382 and 183. Plants were cultured and harvested as described in Table 8.2. Nitrogenase activity is expressed as C_2H_4 produced per plant or nodule biomass.

Table 8.3

Family	Segregant phenotype		$LSD_{0.05}$
	nts[a]	wild-type[b]	
a) nmol C_2H_4 produced. g plant fresh wt.$^{-1}$.min^{-1}			
1007	12.74 (3.56)[c]	0.81 (0.88)	(0.47)
382	7.95 (2.05)[d]	1.10 (0.00)	(0.76)
183	7.26 (1.95)[d]	1.58 (0.41)	(0.78)
b) nmol C_2H_4 produced. g nodule fresh wt.$^{-1}$. min^{-1}			
1007	78 (4.35)[d]	153 (5.02)	(0.26)
382	58 (4.05)[d]	167 (5.07)	(0.48)
183	52	155	28

[a] each entry in the Table is the mean of 9, 7 and 6 plants respectively for families 1007, 382 and 183.

[b] each entry in the Table is the mean of 9, 9 and 4 plants respectively for families 1007, 382 and 183.

[c,d] raw data required either square-root (c) or $\log_e$ (d) transformation to satisfy assumptions for an analysis of variance; means and LSD of transformed data are shown in brackets.

The nodulation characteristics of 1007, 382 and 183 *nts* segregants were very similar. Nodule tissue constituted the same proportion of total plant fresh weight in *nts*382, *nts*1007 and *nts*183 segregants (Table 8.2). Furthermore, the pattern of nodulation was similar in the three selected lines. Mutant segregants had significantly more nodules on the tap root and especially on lateral roots (Table 8.4). The nodulation pattern in nitrate-grown *nts* segregants strikingly contrasted the pattern of nodulation in both N_2-dependent wild-type Bragg and nitrate-grown wild-type siblings. This contrast is illustrated for family 1007 and Bragg in Fig. 8.1.

Consistent with the trend reported in the previous two chapters, 382 *nts* segregants had significantly lower plant fresh weights than did wild-type siblings (Table 8.5). This trend was also evident in prolifically-nodulating 1007 and 183 *nts* segregants (Table 8.5).

8.3.2 Inheritance of *nts*733

In contrast to M_2 families 1007, 382 and 183, M_2 family 733 segregated for three phenotypes. In addition to wild-type, there were two other distinct phenotypes designated *nts* and marginal *nts*. These three nodulation phenotypes are demonstrated in Fig. 8.2. Fig. 8.2(a) shows *nts*733-1 (phenotype = *nts*) alongside a wild-type sibling, and Fig. 8.2(b) illustrates *nts*733-2 (phenotype = marginal *nts*) also with a wild-type sibling. Selection *nts*733-1 was a small plant with 338 nodules and was characterized by prolific nodulation over the entire root system (Fig. 8.2(a)). Marginal selection *nts*733-2 was comparable to the wild-type in overall

Table 8.4: Distribution of nodules on *nts* and wild-type siblings of segregating M_3 families of 1007, 382 and 183. Plants were cultured and harvested as described in Table 8.2. Tap root and lateral root nodule number are expressed per plant biomass.

Table 8.4

Family	Nodule number . g plant fresh wt^{-1}		$LSD_{0.05}$
	nts[a]	wild-type[b]	
	a) on the tap root		
1007	8.6 (2.02)[c]	0.6 (-0.62)	(0.22)
382	9.6 (2.22)[c]	0.7 (-0.45)	(0.20)
183	5.4 (1.66)[c]	1.1 (0.07)	(0.26)
	b) on lateral roots		
1007	59.1 (3.87)[d]	0.9 (0.50)	(0.44)
382	89.2 (9.4)[e]	1.5 (1.1)	(0.4)
183	79.3 (8.9)[e]	3.3 (1.8)	(0.8)

[a] each entry in the Table is the mean of 22, 14 and 8 plants respectively for families 1007, 382 and 183.

[b] each entry in the Table is the mean of 18, 18 and 8 plants respectively for families 1007, 382 and 183.

[c,d,e] raw data required either $\log_e$ (nodule number) (c), $\log_e$ (nodule number + 1) (d) or square-root (nodule number) (e) transformation to satisfy assumptions for an analysis of variance; means and LSD of transformed data are shown in brackets.

Figure 8.1: Nodulation of N_2-dependent Bragg, and of nitrate-grown *nts*1007 and wild-type 1007 from a segregating M_3 family. Plants were harvested 49 days after planting. Bragg plants received N-free nutrients throughout growth, whereas 1007 M_3 segregants received 5mM KNO_3. All plants were inoculated at day 0 and day 4 with *R. japonicum* strain USDA110.

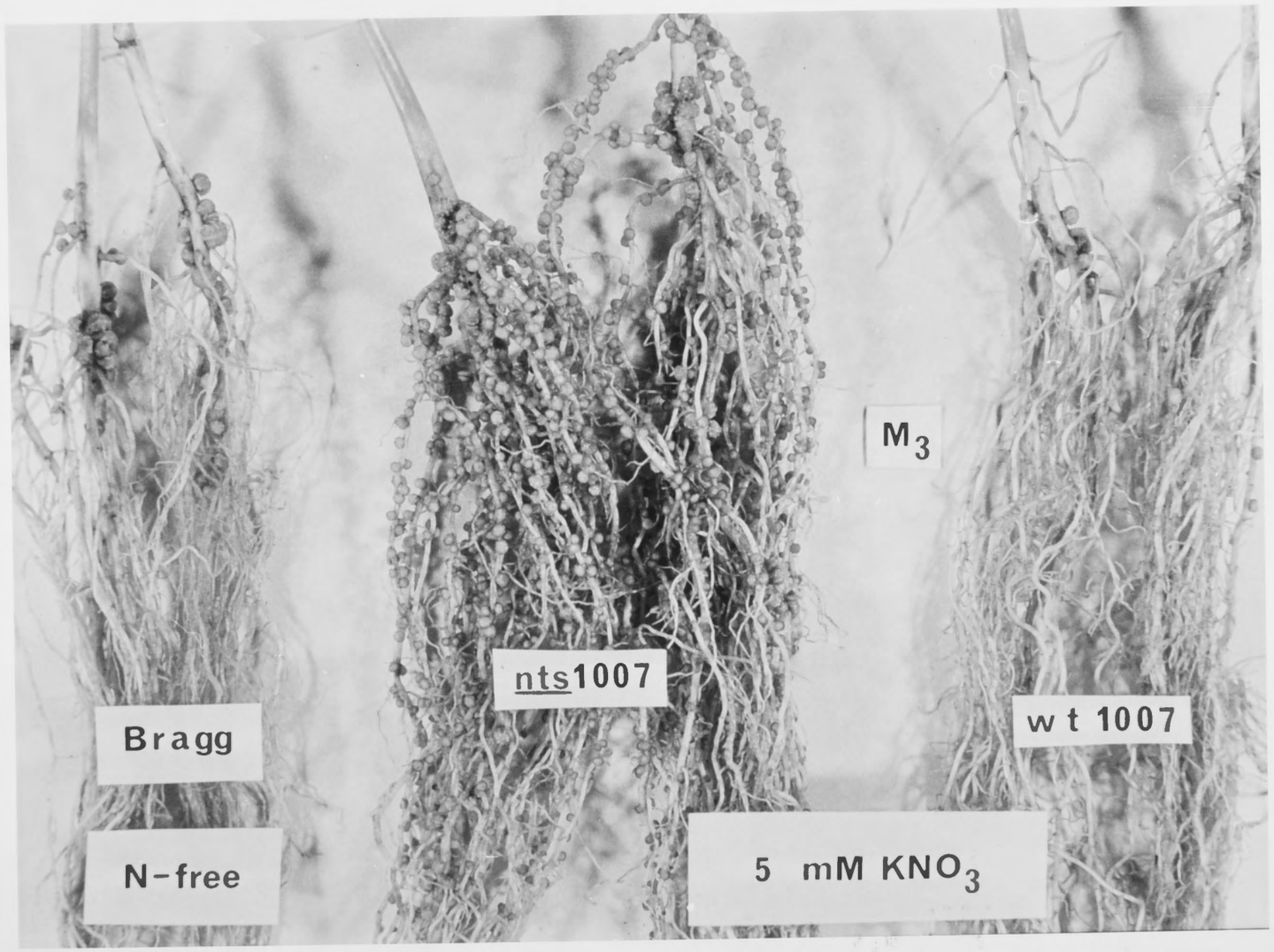

M3
nts1007
wt 1007
Bragg
N-free
5 mM KNO3

Table 8.5: Plant fresh weights of *nts* and wild-type siblings from M_3 segregating families of 1007, 382 and 183. Plants were cultured and harvested as described in Table 8.2.

Table 8.5.

Family	Plant fresh weight (g)		$LSD_{0.05}$
	nts[a]	wild-type[b]	
1007	9.0 (2.96)[c]	13.9 (3.69)	(0.27)
382	8.2 (2.84)[c]	15.2 (3.88)	(0.25)
183	12.3 (3.49)[c]	16.5 (4.03)	(0.39)

[a] each entry in the Table is the mean of 22, 14 and 8 plants respectively for families 1007, 382 and 183.

[b] each entry in the Table is the mean of 18, 18 and 8 plants respectively for families 1007, 382 and 183.

[c] raw data required square-root transformation to satisfy assumptions for an analysis of variance; means and LSD of transformed data are shown in brackets.

Figure 8.2: Nodulation phenotypes in M_2 family 733. The plants were inoculated with *R. japonicum* strain CB1809 (= USDA136), and were harvested 48 days after planting and culture on 5mM KNO_3. a) *nts*733-1 and a wild-type sibling. b) *nts*733-2 (phenotype = marginal *nts*) and a wild-type sibling.

Figure 8.2a)

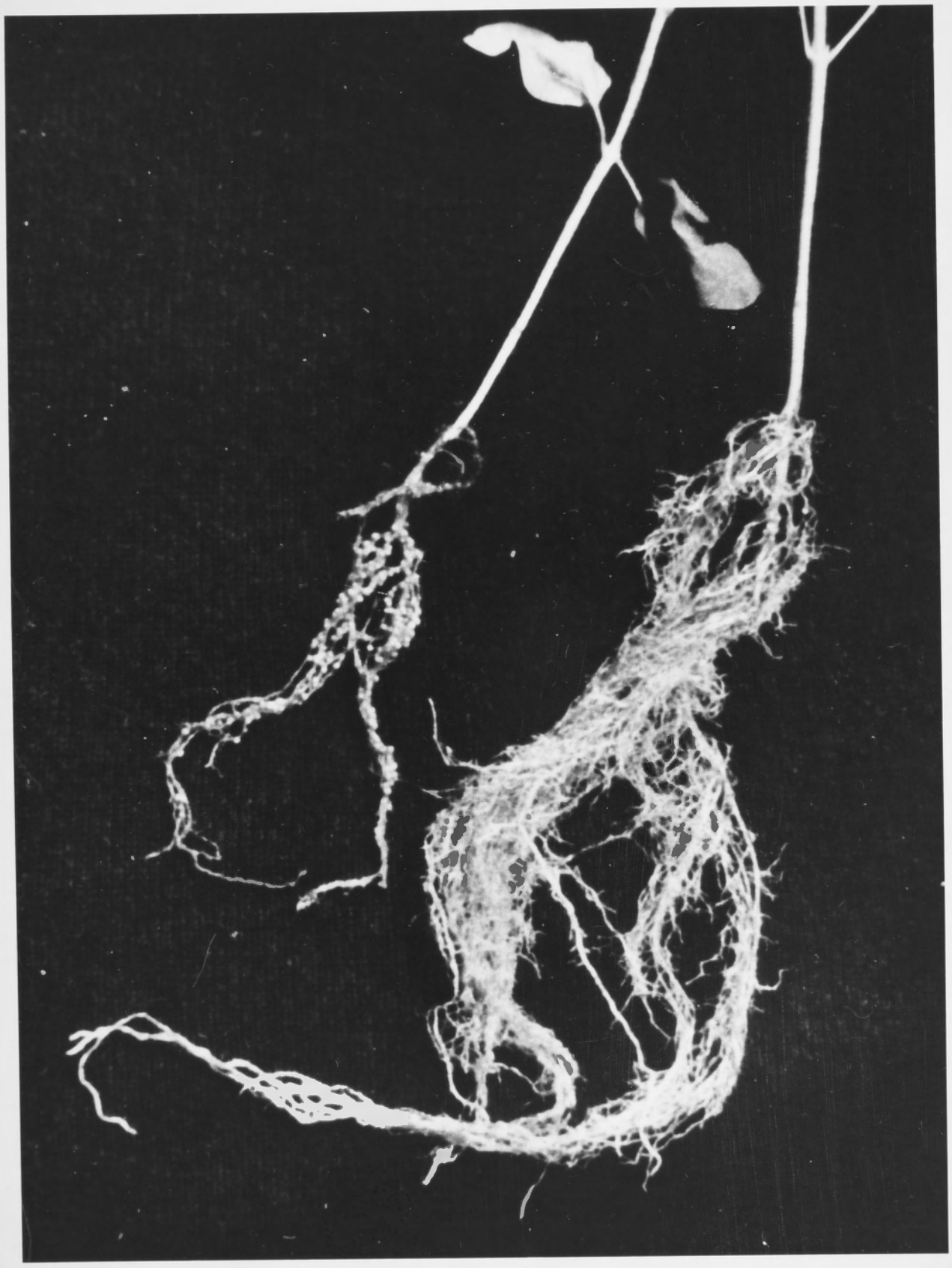
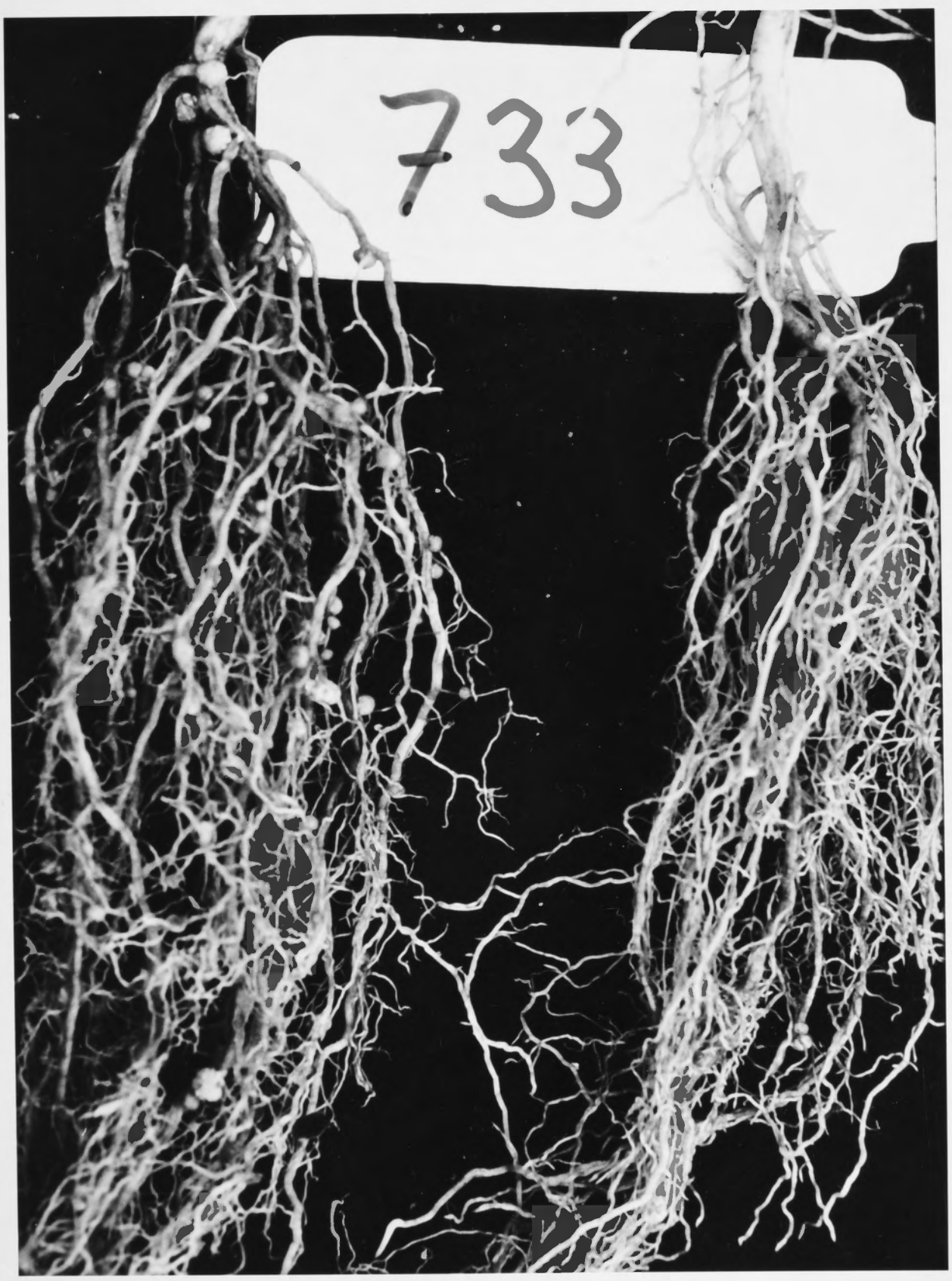


Figure 8.2b)



growth but had more nodules than wild-type siblings. Indeed, *nts*733-2 had 88 nodules whereas wild-type siblings had 18 ± 10 (± S.D.) nodules per plant. The extent of nodulation in the wild-type siblings was very similar to that of Bragg plants cultured under identical conditions and harvested two days earlier. Nitrate-grown wild-type Bragg plants had 19 ± 7 nodules per plant.

Individual plants in M_2 family 733 were allowed to self-fertilize and produced families of M_3 seed. Subsequently, M_3 families (each derived from a single M_2 plant) were cultured on 5mM KNO_3 for 51-63 days and then screened for the *nts* phenotype. All M_3 progeny derived from *nts*733-1 expressed the *nts* phenotype (Table 8.6), indicating that *nts*733-1 was homozygous for the mutated gene. Similarly, M_2 wild-type 733 siblings were pure breeding and only produced wild-type progeny (Table 8.6). In contrast, M_3 progeny derived from marginal selections *nts*733-2 and *nts*733-3 segregated for the nodulation phenotype (Table 8.6). The segregation ratio in M_3 progeny derived from M_2 marginal *nts* plants approximated 1 nts to 2 marginal *nts* to 1 wild-type (chi-square values were not significant) (Table 8.6). Therefore, it is likely that M_2 marginal *nts* plants were heterozygous for the mutated gene and that the *nts*733 character is incompletely dominant.

Table 8.6: Segregation ratios of M_3 progeny derived from M_2 733 plants.

Table 8.6

M_2 selection	M_2 phenotype	Observed M_3 segregation ratio			Expected M_3 segregation ratio[a]			Chi-square[b]
		nts :	marginal *nts* :	wild-type	*nts* :	marginal *nts* :	wild-type	
733-1	*nts*	12	0	0	12	0	0	-
733-2	marginal *nts*	0	6	3	2.25	4.50	2.25	1.61
733-3	marginal *nts*	3	4	5	3	6	3	1.21
733 (2 plants)	wild-type	0	0	30	0	0	30	-

[a] expected segregation ratio if the *nts*733 character is incompletely dominant

[b] Chi-square (0.05 level of significance; 2 degree of freedom) = 5.99. Therefore chi-square values listed in the Table are not significant.

8.4 Discussion

The main conclusions to be drawn from data presented in this chapter are that in the presence of nitrate the *nts* characters in *nts*382, *nts*1007 and *nts*183 are recessive and that the *nts*733 character is incompletely dominant. It is possible that environmental conditions, such as nitrate supply, may affect the phenotype of heterozygotes. Therefore, plants heterozygous for the *nts* mutation are being tested for nodulation in the absence of nitrate.

M_2 families 382, 1007 and 183 segregated for two nodulation phenotypes, namely *nts* and wild-type. Mutant plants were pure-breeding and therefore homozygous for the respective mutation, whereas some M_2 wild-type plants were not pure-breeding and M_3 progeny derived from these plants segregated 1 *nts* mutant to 3 wild-types (Table 8.1). This is the expected segregation ratio for a Mendelian recessive character in progeny derived from a heterozygous plant. As well as the mode of inheritance being the same in *nts*382, *nts*1007 and *nts*183, the nodulation characteristics of these three mutants are very similar (Tables 8.2, 8.3 and 8.4). Furthermore, *nts* segregants from the three families had significantly lower plant fresh weights than did the respective wild-type siblings (Table 8.6). As described in Chapters 6 and 7, *nts*382 is a mutant in the regulation of nodule development, and the extent and pattern of nodulation described in this chapter for *nts*1007 and *nts*183 indicates that these are also mutants in the regulation of nodule development. Complementation tests are being conducted between the three

lines to ascertain whether *nts*382, *nts*1007 and *nts*183 are allelic mutants (Dr. A. Delves, *pers. comm.*).

The data presented here are consistent with only one locus having been mutated in the four *nts* lines. However, soybean is generally considered to be a diploidized tetraploid (based on the basic chromosome number (x) being equal to ten) (Lackey, 1981). There is no direct evidence that indicates that soybean is tetraploid, but there is evidence for considerable genome duplication. For example, despite diploid soybean (2n = 40) having regular bivalent pairing at meiosis, the degree of meiotic chromosome pairing in haploid soybeans (2n = 20) is sufficient to indicate a polyploid cytogenetic composition for diploid soybean (Crane *et al.*, 1982). Therefore, it is plausible that some loci are duplicated in soybean.

The mutant characters rj_1, Rj_2, Rj_3 and Rj_4 are inherited as monogenic mutations (Williams and Lynch, 1954; Caldwell, 1966; Vest, 1970; Vest and Caldwell, 1972). Similarly, it is likely that the *nts* characters in 1007, 382 and 183 are inherited as monogenic recessives. All of the segregating M_3 families from 1007, 382 and 183 M_2 wild-types segregated one mutant to three wild-types (Table 8.1), the expected ratio for a monogenic recessive. If the mutated locus was duplicated, this segregation

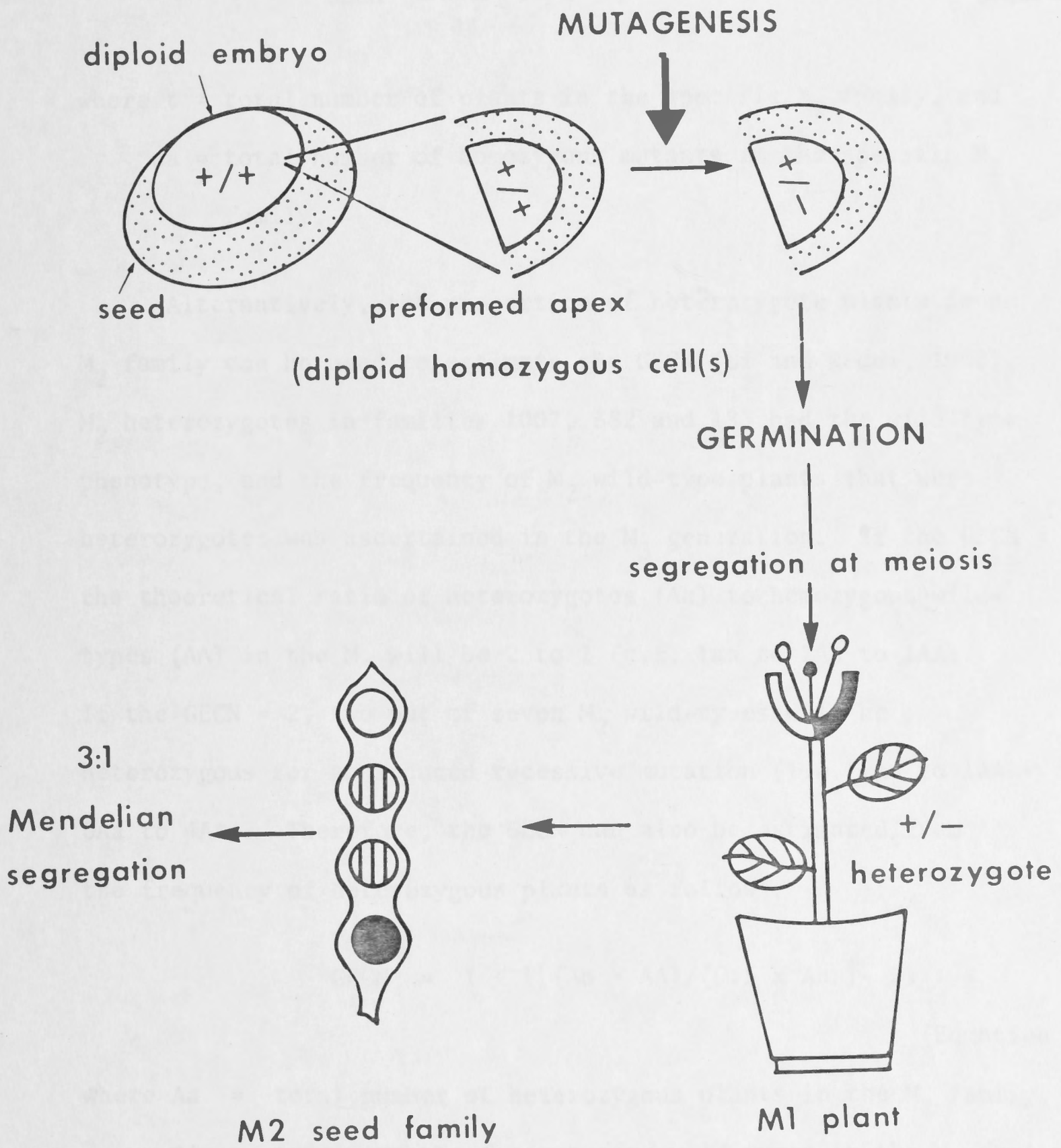
ratio would be likely to occur in 50% of segregating M_3 families (progenitor plants of these M_3 families would have been homozygous for the mutation at one locus and heterozygous at the duplicated locus). The remaining 50% of segregating families would segregate one mutant to 15 wild-types (progenitor plants of these segregating families would have been heterozygous for the mutation at both loci). This latter ratio was not observed in the three segregating 1007 M_3 families (Table 8.1). Therefore, it is likely that the mutated gene was not duplicated in *nts*1007 and that the *nts*1007 character is inherited as a monogenic recessive. Similarly, each of the segregating M_3 families in lines 382 and 183 also segregated one mutant to three wild-types (rather than one to fifteen). Crosses between pure-breeding mutant and parental lines are being conducted to confirm the apparent monogenic inheritance of the mutant characters (Dr. A. Delves, *pers. comm.*).

Assuming that the *nts* phenotypes arose from one mutated locus, the frequency of homozygous mutants and heterozygotes in the M_2 families can be used to estimate the genetically effective cell number (GECN) (Li and Redei, 1969). This is the number of cells in the seed that give rise to the seeds of the next generation, i.e. the number of germline cells in the seed. The GECN is of practical significance in the planning of a mutagenesis and selection program for a particular species, since each germline cell in the seed represents a genetic unit.

If the germline is composed of one cell at the time of mutagenesis (i.e. GECN = 1), homozygous recessive mutants will segregate in the M_2 progeny at the simple Mendelian ratio of 1 mutant to 3 wild-types (Fig. 8.3). If the GECN = 2, homozygous recessive mutants will segregate at a frequency of 1 mutant to

Figure 8.3: Segregation of an induced recessive mutation in a species with a GECN = 1. When the germline is composed of 1 cell at the time of mutagenesis, recessive mutations will segregate in the M_2 generation at the simple Mendelian ratio of 1 mutant to 3 wild-type. If GECN = 2, recessive mutations will segregate at a frequency of 1 mutant to 7 wild-type (i.e. 1 to 3 + 0 to 4) in the M_2. If GECN = 3, the segregation ratio will be 1 to 11 (i.e. 1 to 3 + 0 to 4 + 0 to 4), etc.

GENETICALLY EFFECTIVE CELL NUMBER (GECN)



7 wild-types (i.e. 1 to 3 + 0 to 4) in the M_2. If the GECN = 3, the M_2 segregation ratio for a recessive mutant will be 1 mutant to 11 wild-types (i.e. 1 to 3 + 0 to 4 + 0 to 4) etc. Thus, the frequency of homozygous mutants can be used to calculate the apparent GECN as follows:

$$GECN = \frac{t}{aa} \div 4 \qquad \text{(Equation 1)}$$

where t = total number of plants in the specific M_2 family, and

aa = total number of homozygous mutants in the specific M_2 family.

Alternatively, the proportion of heterozygote plants in an M_2 family can be used to estimate the GECN (Li and Redei, 1969). M_2 heterozygotes in families 1007, 382 and 183 had the wild-type phenotype, and the frequency of M_2 wild-type plants that were heterozygotes was ascertained in the M_3 generation. If the GECN = 1, the theoretical ratio of heterozygotes (Aa) to homozygous wild-types (AA) in the M_2 will be 2 to 1 (c.f. 1aa to 2Aa to 1AA). If the GECN = 2, two out of seven M_2 wild-types will be heterozygous for an induced recessive mutation (i.e. 2Aa to 1AA + 0Aa to 4AA). Therefore, the GECN can also be estimated from the frequency of heterozygous plants as follows:

$$GECN = 1 + \{[(Aa + AA)/(0.5 \times Aa)] - 3\} \div 4 \qquad \text{(Equation 2)}$$

where Aa = total number of heterozygous plants in the M_2 family, and

AA = total number of homozygous wild-types in the M_2 family.

If the proportion of heterozygotes is high and the apparent GECN is less than one (due to a selection pressure against the homozygous wild-type or to random variation in class sizes) the

above Equation 2 does not hold, and the apparent GECN in this situation can be calculated as follows:

$$GECN = \frac{Aa + AA}{0.5 \times Aa} \div 3 \qquad \text{(Equation 3)}$$

Using the above equations (which assume that the mutated gene is not duplicated) the apparent GECN in M_1 seeds 1007, 382, 183 and 733 was calculated and are listed in Table 8.7. The GECN was estimated from both the frequency of homozygotes in the M_2 (column 3 of Table 8.7) and the ratio of heterozygotes to homozygous wild-types in the M_2 (column 5 of Table 8.7). The latter estimate for families 1007, 382 and 183 was calculated from the frequency of segregating M_3 families derived from M_2 wild-type plants. In contrast to *nts*1007, *nts*382 and *nts*183, *nts*733 heterozygotes were distinguishable from homozygous wild-types in the M_2 and, therefore, this estimate was calculated on the ratio of marginal *nts* variants to wild-types in the M_2 generation.

The two estimates of the GECN for each individual family were approximately the same (compare columns 3 and 5 of Table 8.7), and assuming monogenic inheritance, the mean GECN over the four mutant families was 3.4. The value of the GECN is considered to range from one to ten, depending on the species (Redei, 1975); for example, the GECN is one for coffee (*Coffea arabica* L.) (Moh, 1961), two for *Arabidopsis thaliana* L. (Li and Redei, 1969) and five or more for *Zea mays* L. (Anderson *et al.*, 1949; Johri and Coe, 1983).

Table 8.7: Estimation of the genetically effective cell number (GECN) from the frequency of M_2 homozygous mutants and M_2 heterozygotes (assuming monogenic inheritance). The mean apparent GECN over the four families was 3.4.

Table 8.7

Family	aa/t [a]	GECN [b]	Aa/(aA + AA) [c]	GECN [d]	Mean GECN [e]
1007	6/48	1.5	3/23	4.1	2.8
382	2/17	2.1	1/6	2.3	2.2
183	3/8	0.7	1/1	0.7	0.7
733	1/38	9.5	2/37	6.4	8.0

[a] aa = the number of homozygous mutants in the M_2 family and t = the total number of plants in the M_2 family.

[b] estimated from the frequency of homozygous mutants in the M_2 (listed in column 2) using Equation 1 (see text).

[c] Aa = the number of M_2 heterozygotes and (Aa + AA) = the number of heterozygotes plus the number of homozygous wild types.

[d] estimated from the ratio of Aa : (Aa + AA) using Equation 2 or 3 (as described in text).

[e] mean of the two estimates described above.

As stated above, in estimating the GECN it was assumed that only one mutated locus in each of the selected lines gave rise to the respective phenotypes. If this was not the case and two loci were mutated in the selected lines, the frequency of M_2 homozygous mutants could be used to calculate the apparent GECN as follows:

$$GECN = \frac{t}{aa} \div 16 \qquad \text{(Equation 4)}$$

where t and aa are the total number of plants and the number of homozygous mutants in the M_2 family, respectively.

Thus, assuming bigenic inheritance and using Equation 4 the apparent GECN for 1007, 382, 183 and 733 would be 0.5, 0.5, 0.2 and 2.4, respectively. Unless diplontic selection is occurring against the mutant or wild-type allele, the apparent GECN cannot be significantly less than one. The observed segregation ratios in M_3 families of 1007, 382 and 183 closely approximated 1 mutant to 3 wild-types, indicating that there were not significant discrepancies in the fitness of mutant and wild-type gametes and in the development of mutant and wild-type embryos in the developing pod. Since there was no selection in favour of the mutant allele, GECN estimates discount bigenic inheritance of the mutant character in *nts*382, *nts*1007 and *nts*183. The segregation data for *nts*733 are consistent with the *nts* phenotype being conditioned by one mutated locus, but the large difference in the apparent GECN between family 733 and the other three families emphasizes the need for traditional genetic analysis.

The GECN is of practical significance because it can be used to estimate the recovery rate of mutants (Redei, 1975). The probability of recovering an existing mutant in an M_2 family is

dependent on the number of plants screened from this family, the nature of inheritance of the mutation and the GECN. These concepts are illustrated in Table 8.8 (for recessive mutations) and in Table 8.9 (for dominant or incompletely dominant mutations). Obviously, the probability of recovering an existing mutant from an M_2 family increases with the number of plants screened. However, for a specific number of plants screened per M_2 family, the probability of detecting a mutant (if it exists) decreases as the GECN increases (Table 8.8 and 8.9) (also, see Redei, 1975).

In this study, 12 seedlings per M_2 family were screened for the *nts* phenotype. Using GECN = 3.4, it is likely that only 50 to 70% of existing recessive *nts* mutants were recovered (Table 8.8), whereas the majority (approximately 90%) of dominant or incompletely dominant mutants would have been recovered (Table 8.9).

To my knowledge, there is no existing estimate of the GECN for soybean in the literature. The EMS-mutagenized population derived from this study (Chapter 3) has been maintained, and the estimate of the GECN described above will be of value in screening programs for other mutants that may be scientifically or agronomically useful. This discussion is also relevant to mutagenized soybeans that exist as the bulk M_2 populations. For example, using Table 8.8 and the GECN estimate described above, it is likely that approximately 50 to 70% of recessive mutants existing in a bulk M_2 population will be recovered from x M_2 plants screened, where x is 12 times the number of M_1 plants that produced seed and contributed to the M_2 population.

Table 8.8: The probability of recovering an existing recessive mutant in a M_2 family. Each entry in the Table is the probability of finding an existing mutant for a specific GECN (effective cell number) and a specific number of plants screened in the M_2 family.

Table 8.8.

Plants screened per M_2 family	GECN 1	2	3	4	5	6	10
1	0.250	0.125	0.083	0.063	0.050	0.042	0.025
2	0.438	0.234	0.160	0.121	0.098	0.082	0.049
4	0.684	0.414	0.294	0.228	0.185	0.157	0.096
8	0.900	0.656	0.501	0.403	0.337	0.289	0.183
12	0.968	0.799	0.648	0.539	0.460	0.400	0.262

Table 8.9: The probability of finding an existing dominant or incompletely dominant mutant in an M_2 family. Each entry in the Table is the probability of detecting an existing mutant for a specific GECN and a specific number of plants screened in the M_2 family.

Table 8.9.

Plants screened per M_2 family	GECN 1	2	3	4	5	6	10
1	0.750	0.375	0.250	0.188	0.150	0.125	0.075
2	0.938	0.609	0.438	0.340	0.278	0.234	0.144
4	0.996	0.847	0.684	0.564	0.478	0.414	0.268
8	1.000	0.977	0.900	0.810	0.728	0.656	0.464
12	1.000	0.996	0.968	0.917	0.858	0.799	0.608

Prior to publication of the results presented in this chapter, the nature of inheritance of the mutant characters will be confirmed by traditional genetic analysis. Nevertheless, *nts*382, *nts*1007 and *nts*183 are similar to the one nitrate-tolerant pea mutant nod_3 (Jacobsen and Feenstra, 1984) in that the mutant character is inherited as a recessive. Mutant line *nts*733 is unique because the *nts* character is incompletely dominant in this line.

CHAPTER NINE

ISOLATION AND INITIAL CHARACTERIZATION OF THE CONSTITUTIVE NITRATE REDUCTASE-DEFICIENT MUTANTS NR328 AND NR345.

9.1 Introduction

Studies on legume mutants have shown that alteration in the regulation of nodule development (Jacobsen and Feenstra, 1984; Chapters 6 and 7) or impairment in nitrate utilization (Feenstra *et al.*, 1982) can result in increased nitrogen fixation in the presence of nitrate. The successful approach described in Chapter 6 involved selection for nodulation on nitrate and this made it feasible to recover mutants in both of these categories. As an alternative to screening for nodulation on nitrate, plants can be selected directly for the inability to utilize nitrate (Feenstra and Jacobsen, 1980).

In legume-*Rhizobium* root nodule symbioses, the plant moiety is responsible for the assimilation of ammonium (Dilworth, 1980) and therefore the appropriate nitrate non-utilizing mutant must be anomalous in nitrate utilization prior to ammonium formation. The three major processes that occur prior to ammonium formation are nitrate uptake (1), nitrate reduction by nitrate reductase (2) and nitrite reduction by nitrite reductase (3) (Beevers and Hageman, 1983):

$$NO_3^- \xrightarrow{(1)} NO_3^- \xrightarrow{(2)} NO_2^- \xrightarrow{(3)} NH_4^+$$

(exogenous) (endogenous)

Nitrite is highly reactive and generally does not accumulate in plant tissue (Srivastava, 1980), and therefore a mutant which lacks nitrite reductase (and accumulates nitrite) would be inappropriate. On the other hand, mutations that condition decreased nitrate uptake or nitrate reduction are less likely to impose deleterious pleiotrophic effects on plant growth.

Nitrate reductase (NR) - deficient mutants have been isolated in several higher plant species (for review see Kleinhofs *et al.*, 1983 and Harper *et al.*, 1985). However, nitrate uptake mutants have only been isolated in *Arabidopsis thaliana* L. (Braaksma, 1982). Efficient procedures for assaying NR (Kleinhofs *et al.*, 1978) and the availability of chlorate as a positive selection agent have aided in the isolation of these mutants. Chlorate is an analogue of nitrate and, although it may have other effects (Cove, 1976), it is generally considered toxic due to the catalytic conversion of chlorate to chlorite by NR (Liljeström and Aberg, 1966; Hofstra, 1977).

In legumes, nitrate metabolism mutants have been isolated using both chlorate resistance (Feenstra and Jacobsen, 1980; Nelson *et al.*, 1983) and an *in vivo* NR assay (Kleinhofs *et al.*, 1978) as screening tools. Pea mutant E_1 has decreased NR activity and was selected on the basis of chlorate resistance (Feenstra and Jacobsen, 1980). Also in pea, mutants A317, A334 and A300 are NR-deficient but these lines were selected using an *in vivo* NR screen (Kleinhofs *et al.*, 1978). Nitrate uptake was not impaired in these pea mutants and nitrate accumulated as a result of decreased nitrate reduction (Feenstra and Jacobsen, 1980; Warner *et al.*, 1982). E_1 is the only pea mutant that has been used to study nitrate inhibition of nodulation and nitrogen fixation (Feenstra *et al.*, 1982; Jacobsen, 1984).

NR mutants have also been isolated in soybean (Nelson *et al.*, 1983). However, in contrast to pea (Lahav *et al.*, 1976) and most other higher plants that have been studied, there is

more than one NR activity in soybean (Harper *et al.*, 1985). Generally, NR activity is only expressed when nitrate is present in the growth medium (Srivastava, 1980; Beevers and Hageman, 1983). Soybean is an exception to this generality and Harper (1974) showed that leaf tissue from N_2-dependent plants (i.e. grown with *Rhizobium japonicum* and without combined nitrogen) expressed NR activity. Similarly, it was demonstrated that leaf tissue from urea-grown plants had NR activity (Lahav *et al.*, 1976). Since this activity did not require nitrate to be present, it was termed constitutive or non-inducible NR (Harper *et al.*, 1985). Constitutive NR (cNR) activity is not expressed in soybean roots (Kakefuda *et al.*, 1983; Nelson *et al.*, 1983) or soybean cell cultures (Nelson *et al.*, 1984), and NR activity in these tissues is dependent on nitrate being present. Thus, in addition to cNR, there is also nitrate-inducible NR (iNR) activity in soybean.

Multiple soybean NR activities have also been biochemically distinguishable. Using column chromatography, Jolly *et al.* (1976) and Campbell (1976) separated two NR activities from young unifoliolate leaves of nitrate-grown soybean seedlings. The two activities differed in affinities for nitrate and for the reduced pyridine nucleotides NADH and NADPH. These NR activities were also identified in cotyledons of nitrate-grown seedlings (Orihuel-Iranzo and Campbell, 1980).

Genetic evidence for the presence of multiple soybean NR activities has been reported by Nelson *et al.* (1983). They isolated three allelic chlorate-resistant mutants (LNR-2, LNR-3 and LNR-4) (Ryan *et al.*, 1983) that lacked cNR, but expressed iNR in root

and leaf tissue. Subsequent studies on the parent cultivar Williams and the cNR mutant nr_1 (formerly LNR2) led to the identification of three biochemically distinct nitrate reductases in soybean, and to the correlation of these activities with cNR and iNR (Streit *et al.*, 1985). Urea-grown wild-type plants contained two constitutive nitrate reductases, designated c_1NR and c_2NR. The c_1NR used both NADPH and NADH, whereas c_2NR used only NADH as an electron donor. The two forms were also distinguishable in the apparent K_m for nitrate, but otherwise had similar pH optima (6.5), sedimentation coefficients and electrophoretic mobilities. Only one iNR was identified in nitrate-grown nr_1 plants (that lacked cNR). This iNR was distinguishable from c_1NR and c_2NR in that it had a higher pH optimum (7.5), a higher sedimentation coefficient and was less mobile on an electrophoretic gel. Like c_2NR, iNR only used NADH as an electron donor (Streit *et al.*, 1985).

Although cNR is responsible for 12.5 to 20% of total measurable NR activity in soybean (Harper *et al.*, 1985), the nr_1 mutation did not impede nitrate utilization or confer nitrate tolerance to symbiotic nitrogen fixation (Ryan *et al.*, 1983). Therefore, plausible target mutations for impeding nitrate utilization and concomitantly increasing nitrogen fixation are those that decrease either nitrate uptake or iNR activity. However, the nitrate uptake system in soybean has not been characterized and the prospect of isolating an uptake mutant is not encouraging since Nelson *et al.* (1983) did not recover this type of mutant from a screen for chlorate resistance (chlorate is considered to act as an analogue for nitrate uptake, as well as for nitrate reduction (Deane-Drummond and Glass, 1982)). Furthermore, there are inherent difficulties in selecting directly for an iNR deficiency. It is likely

that decreased iNR activity in leaf tissue would be masked by the presence of cNR activity. And although cNR is not expressed in root tissue (Nelson *et al.*, 1983), the concept of assaying this tissue for iNR activity violates the requirement that the screening procedure be rapid. Similarly, cell cultures only express iNR (Nelson *et al.*, 1984), but soybean cell cultures are thus far recalcitrant to reliable plant regeneration from established cell lines.

In view of the difficulty of screening directly for iNR mutants, a feasible approach to obtain mutants with decreased nitrate utilization (for increased nitrogen fixation) is to select firstly for cNR mutants. Depending on the existance of common processes that are specifically required for both cNR and iNR activity, some cNR mutants may also be affected in iNR activity. Alternatively, cNR-deficient lines can serve as parent material for further mutagenesis and screening for iNR activity.

In this chapter, a rapid screening assay for *in vivo* cNR in unifoliolate leaves is described. Using this procedure, two cNR mutants were obtained and characterized for *in vivo* NR activity and for nodulation in the presence of nitrate.

9.2 Materials and Methods

9.2.1 Plant material. EMS-derived M_2 families of parent cultivar Bragg (Chapter 3) were screened for cNR mutants. For comparison, Williams and nr_1 (formerly LNR-2) (Nelson *et al.*, 1983) were included in some experiments. Seeds of nr_1 were obtained from Dr. S. Ryan (CSIRO, Canberra).

9.2.2 Plant culture. All experiments were conducted in the glasshouse. Glasshouse temperatures were kept between 14°C and 30°C and incandescent bulbs supplemented solar light such that the photoperiod was 16h. M_2 seeds were planted 1cm below the surface in sand trays (63cm long, 23cm wide and 6.5cm deep). The trays were inoculated with *R. japonicum* strain CB1809 and saturated with N-free nutrient solution (Chapter 2) twice a week and received tap water every other day. (The plants were inoculated because they were also screened for nodulation; see Chapter 10). Ten to sixteen days after planting, unifoliolate leaves were screened for cNR activity (see below). M_2 variants were saved to produce M_3 progeny, and this progeny was screened for cNR activity as described below. The stability of the mutant characters was also verified in M_4 and M_5 seedlings, except that these plants were cultured in pots (15cm diam.) of vermiculite. M_4 plants were also characterized for *in vivo* NR activity after culture on nitrate, and for nodulation on various nitrogen sources. In these experiments, plants were inoculated with *R. japonicum* strain CB1809 and cultured in pots (15cm diam.) of river sand (2 plants per pot). The pots were watered daily with 600mls of nutrient solution. KNO_3 (2.5mM) and urea (2.5mM, i.e. 5mM N) were added to the nutrient solution as required.

9.2.3 *In vivo* cNR screen. One unifoliolate leaf disc (4.5mm diam.) was removed from each M_2 seedling and screened for cNR activity in a micro-well of a micro-test plate (96F with lid, Nunc (Inter Med), Denmark). Each micro-plate contained 96 wells. Prior to sampling the leaf discs, 130µl of NR assay solution (containing 50mM KNO_3) (Nicholas *et al.*, 1976a) was dispensed into each well using an eight-tip micro-pipette (50-200µl, serial no. 10379, Titertek). Leaf discs were removed from seedlings and placed in the assay solution and the plates were then incubated at 28°C for three to four hours. Subsequently, the degree of cNR activity was detected by adding 130µl of a 1:1 solution of 1% sulfanilamide in 3N HCl and 0.02% N-naphthyl-ethylene diamine HCl in distilled water to each well. This solution turns pink (absorbance maximum - 540nm) in the presence of nitrite (Warner and Kleinhofs, 1974), and therefore absence of or decreased pink colouration in the micro-well assay indicated decreased cNR activity (Fig. 9.1). This screening method was very rapid and inexpensive, and 600 seedlings could be easily screened by one person in a day.

9.2.4 *In vivo* NR assay. *In vivo* NR activity was determined by a modification of the assay described by Nicholas *et al.* (1976a). Six leaf discs (4.5mm diam.) were sampled from unifoliolate leaves and placed in chilled test tubes containing 5mls of NR assay solution. The assay solution was the same as that used by Nicholas *et al.* (1976a) and contained either 0 or 50mM KNO_3. The tubes were incubated in the dark in a 30°C water bath and samples were removed over a 50min incubation period for nitrite determination (Warner and Kleinhofs, 1974). The data were expressed as nmol NO_2^- produced. leaf $disc^{-1}.h^{-1}$ or µmol $NO_2^-.g$ fresh $weight^{-1}.h^{-1}$. Each leaf disc weighed approximately 2.7mg (fresh weight).

9.2.5 Statistics. Data were analysed by analysis of variance, using the general statistical program Genstat (Alvey *et al.*, 1977).

9.3 Results

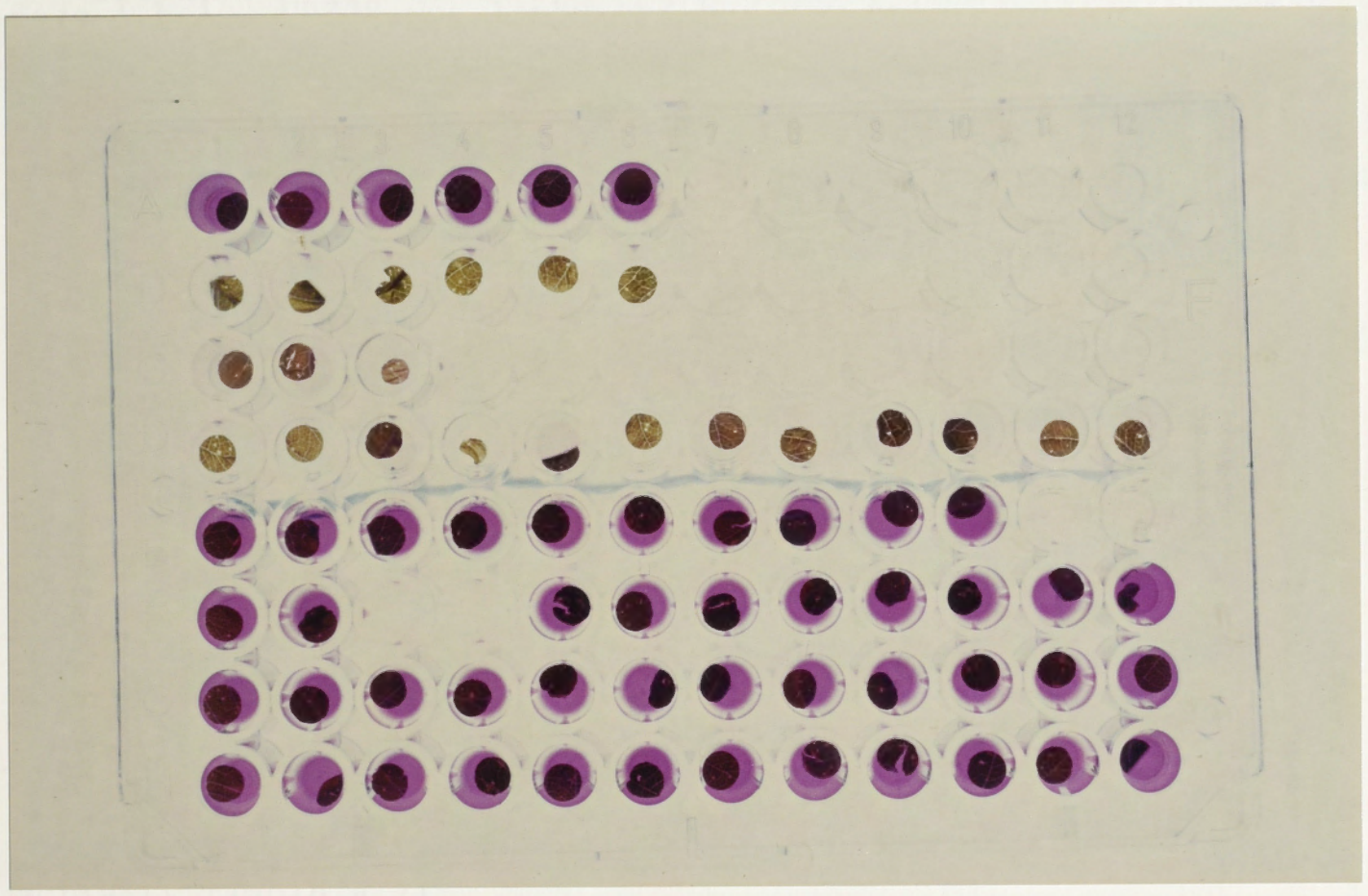
9.3.1 Selection and stability of cNR mutants NR328 and NR345.

Approximately 10,000 seedlings were screened for cNR activity. These plants were derived from 1428 M_2 families; 868 families came from EMS-population 1 (which had 0.9% chlorophyll-deficient plants in the M_2; see Chapter 3) and the remaining 560 families were from EMS-population 2 (2.8% chlorophyll-deficient plants in the M_2). Ten seeds per M_2 family of population 1 were planted, whereas only five seeds were planted for each M_2 family in population 2, and approximately 7,550 and 2,450 M_2 plants were screened in population 1 and population 2, respectively. Thus, about three times as many M_2 plants were screened in population 1 than in population 2.

Several M_2 families segregated for decreased cNR activity. However, based on the rapid screening assay, only two M_2 variants produced M_3 progeny that had little or no cNR activity. M_3 progeny from other M_2 selections were cNR positive and were indistinguishable from Bragg in the rapid screening assay.

The two confirmed cNR-deficient mutants were derived from M_2 families 328 and 345, which belonged to population 2. In each of these families, one out of the five plants screened was cNR-deficient. These variant plants were designated NR328 and NR345. All three M_3 plants derived from M_2 selection NR328 had decreased cNR activity. Similarly, NR345 was pure-breeding and all M_3 progeny (totalling 12 plants) lacked cNR activity. The results of this M_3 screen are illustrated in Fig. 9.1. Bragg

Figure 9.1: Rapid screening cNR assay on Bragg, nr_1 and M_3 progeny of NR328 and NR345. For assay procedures see Materials and Methods. Each micro-well contained a unifoliolate leaf disc from a 14 day-old plant that had been cultured on N-free nutrients. Row 1 = Bragg; row 2 = nr_1 (formerly LNR-2); row 3 = NR328; row 4 = NR345 and rows 5-8 = Bragg.



had considerable cNR activity as indicated by the deep pink colouration in micro-wells containing a Bragg leaf disc. In contrast, micro-wells with an nr_1, NR328 or NR345 leaf disc had almost no colour (Fig. 9.1), indicating that these lines had decreased cNR activity.

In vivo NR assays were also conducted on the plant material illustrated in Fig. 9.1, and the results are listed in Table 9.1. Data are expressed in nmol NO_2^- produced. unifoliolate leaf $disc^{-1}.h^{-1}$. NR328 was leaky and had cNR activity significantly less than Bragg, but significantly more than either NR345 or nr_1 (Table 9.1). Activity in NR328 was 16.2% that of Bragg, whereas NR345 and nr_1 had negligible activity and were only 3.5% and 3.2%, respectively, as active as Bragg in cNR activity (Table 9.1).

In vivo cNR activity was also determined in unifoliolate leaves of M_4 progeny (Table 9.2), and the results obtained were very similar to the M_3 data. Mutants NR345 and nr_1 had minute activity compared to the respective parent cultivars Bragg and Williams, whereas NR328 was leaky and had 14.1% of the wild-type activity (Table 9.2).

The stability of the mutant characters was also verified in the M_5 generation. The results of a rapid screening assay on M_5 NR328 and NR345 material are shown in Fig. 9.2.

9.3.2 *In vivo* NR activity in nitrate-grown plants

Bragg, NR328 and NR345 plants were grown on nitrate and unifoliolate leaves were assayed for NR activity 21 days after planting. These plants were grown during the winter in the glasshouse, and although the first trifoliolate leaf had developed it was fully closed. NR activity was determined without nitrate

Table 9.1: *In vivo* cNR activity of Bragg, nr_1 and M_3 progeny of NR328 and NR345. Unifoliolate leaves were assayed (+ NO_3 assay) 16 days after planting and culture in sand trays. The trays were watered with N-free nutrient solution as described in Materials and Methods. Each entry in the table is the mean of three or four plants.

Table 9.1

Genotype	NR activity		$\log_e$ (NR activity)[a]
	nmol NO_2^- . $disc^{-1}$. h^{-1}	% of Bragg	
Bragg	15.7	100	2.74
NR328	2.6	16.2	0.90
NR345	0.6	3.5	-0.88
nr_1	0.5	3.2	-0.60

[a] raw data required $\log_e$ transformation to satisfy assumptions for analysis of variance. Means of transformed data are shown in column 4. The $LSD_{0.05}$ (based on transformed data) was 0.48.

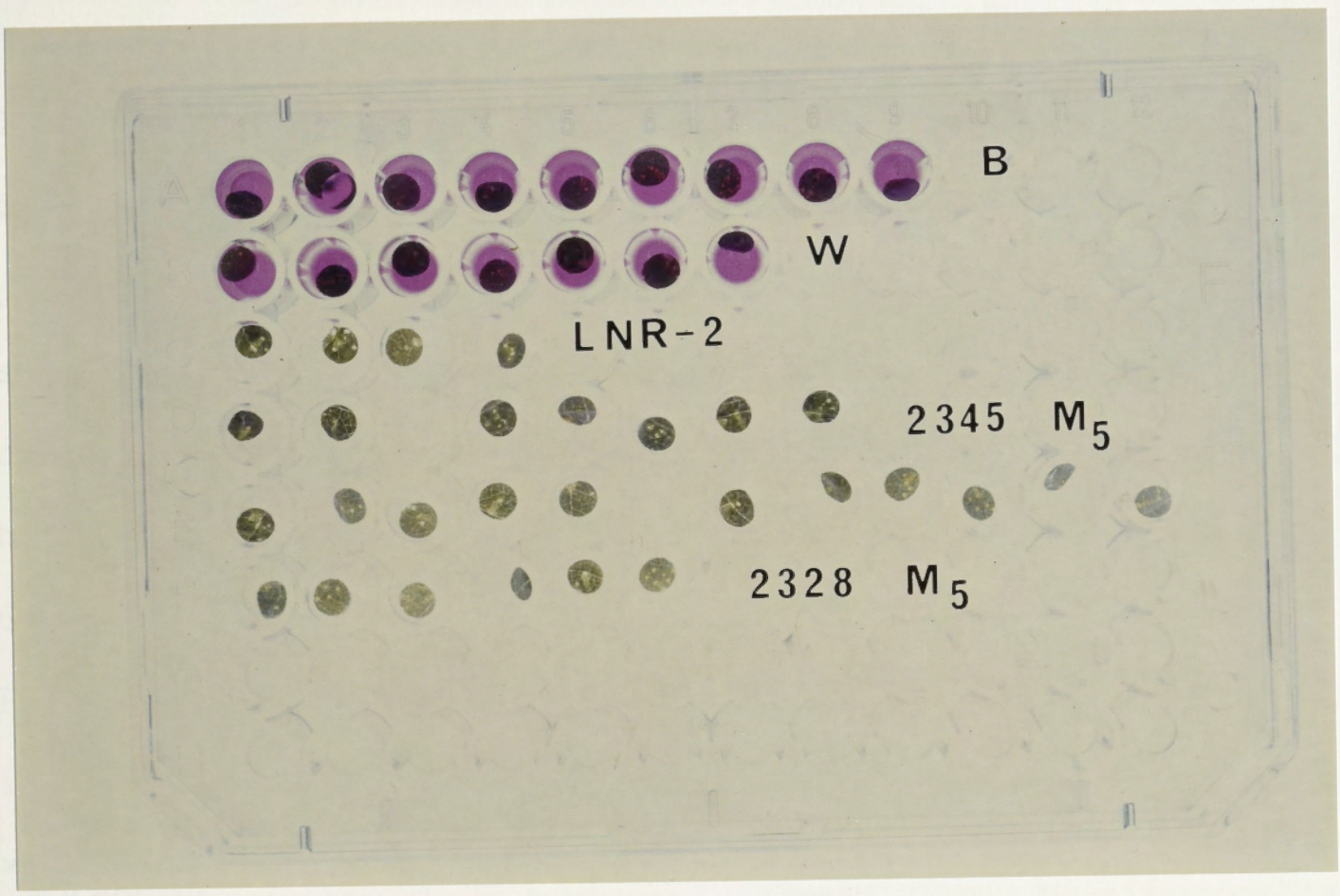
Table 9.2: *In vivo* cNR activity of Bragg, Williams, nr_1 and M_4 progeny of NR328 and NR345. Unifoliolate leaves were assayed 14 days after planting and culture in pots of vermiculite. The pots were watered with N-free nutrient solution. Each entry in the table represents the mean of three to seven plants.

Table 9.2

Genotype	NR activity		
	μmol NO_2^- .g plant fresh weight^{-1} .h^{-1}	% of parent cultivar	$\sqrt{(\text{NR activity})}$[a]
Bragg	9.2	100	3.02
NR328	1.4	14.1	1.09
NR345	0.4	4.3	0.62
Williams	15.0	100	3.86
nr_1	0.3	2.0	0.52

[a] raw data required square-root transformation to satisfy assumptions for analysis of variance. Means of transformed data are shown in column 4. The $LSD_{0.05}$ (based on transformed data) was 0.41.

Figure 9.2: Rapid screening cNR assay on Bragg, Williams, nr_1 (formerly LNR-2) and M_5 progeny of NR328 and NR345. For assay procedures see Materials and Methods. Each well contained a unifoliolate leaf disc from a 13 day-old plant grown on N-free nutrients. Row 1 = Bragg; row 2 = Williams, row 3 = nr_1 (listed as LNR-2 on figure); row 4 = NR345 (listed as 2345 on figure) and rows 5 and 6 = NR328 (listed as 2328 on figure).


B
W
LNR-2
2345 M5
2328 M5

and with 50mM KNO_3 in the assay solution. Both of the cNR mutants expressed iNR activity (Table 9.3). When NR activity was dependent on endogenous nitrate in the leaf tissue ($-NO_3^-$ assay), activity was the same in Bragg, NR328 and NR345 (Table 9.3). However, with 50mM KNO_3 added to the assay solution ($+ NO_3^-$ assay), Bragg NR activity was significantly higher than the activity in either NR328 or NR345 (Table 9.3).

9.3.3 Inhibition of nodulation by combined nitrogen.

A preliminary experiment was conducted to ascertain the inhibitory effect of nitrate and urea on nodulation of parent cultivars and cNR mutants. Table 9.4 shows nodule number . g plant fresh weight^{-1} after 7 weeks culture on N-free, KNO_3-supplemented or urea-supplemented nutrients. Two-way analysis of variance indicated that the effect of combined nitrogen was highly significant (calculated $F = 135.4 > F_{2,65}(0.05) \simeq 3.15$). Genotypic differences were marginally significant (calculated $F = 2.87 > F_{4,65}(0.05) \simeq 2.53$). However, there was no significant interaction between genotype and nitrogen source (calculated $F = 0.95 > F_{8,65}(0.05) \simeq 2.10$), indicating that nodule number in the different genotypes was similarly affected by combined nitrogen. Essentially the same trend was observed for mg nodule fresh weight .g plant fresh weight^{-1} (Table 9.5). The effect of nitrogen source was highly significant (calculated $F = 475.9 > F_{2,67}(0.05) \simeq 3.15$). There was also a significant genotype effect (calculated $F = 10.99 > F_{4,67}(0.05) \simeq 2.53$) and nodule fresh weight per plant biomass was consistently lower in NR328 than in Bragg (Table 9.5).

Table 9.3: *In vivo* NR activity of nitrate-grown Bragg and M_4 progeny of NR328 and NR345. Unifoliolate leaves were assayed 21 days after planting and culture in pots of river sand. The pots were inoculated with *R. japonicum* strain CB1809 and watered daily with 2.5mM KNO_3-supplemented nutrient solution. Leaf tissue was assayed with and without 50mM KNO_3 in the assay solution. Each entry in the table is the mean of six to eight plants. NR activity is expressed as $\mu mol\ NO_2^-.g\ fresh\ weight^{-1}.h^{-1}$.

Table 9.3

Genotype	NR activity	
	$-NO_3^-$ assay[a]	$+NO_3^-$ assay[b]
Bragg	9.1	18.2
NR328	8.9	14.1
NR345	8.9	11.8

[a] no significant difference between genotypes

[b] F-statistic was significant and $LSD_{0.05} = 3.7$.

Table 9.4: Effect of KNO_3 and urea on nodule number .g plant fresh weight^{-1} in Bragg, Williams, nr_1 and M_4 progeny of NR328 and NR345. Plants were harvested 7 weeks after planting and culture in pots of river sand. The pots were inoculated with *R. japonicum* strain CB1809 and watered daily with 600mls of nutrient solution. KNO_3 (2.5mM) and urea (2.5mM) were added to the nutrient solution as required. Each entry in the Table is the mean of three to six plants. Raw data required $\log_e$ transformation to satisfy assumptions for a two-way analysis of variance. Nitrogen source and genotype effects were significant but there was no significant interaction between genotype and N source (see text).

Table 9.4

Genotype	Nodule number .g plant fresh weight^{-1}		
	N-free	2.5mM KNO_3	2.5mM urea
Bragg	6.5	2.9	0.9
NR328	5.1	2.5	0.7
NR345	10.1	2.7	0.7
Williams	5.1	2.2	0.9
nr_1	5.3	1.7	0.9

Table 9.5: Effect of KNO_3 and urea on nodule fresh weight (mg) . g plant fresh weight^{-1} in Bragg, Williams, nr_1 and M_4 progeny of NR328 and NR345. For details of plant culture and harvest see legend to Table 9.4. Raw data required square-root transformation to satisfy assumptions for a two-way analysis of variance. Nitrogen source and genotype effects were significant, but there was no interaction between nitrogen source and genotype; i.e. the genotypes were not differentially sensitive to inhibition of nodule growth by combined nitrogen (see text).

Table 9.5

Genotype	mg nodule fresh weight .g plant fresh weight^{-1}		
	N-free	2.5mM KNO_3	2.5mM urea
Bragg	69.4 (100)[a]	29.0 (42)	6.7 (10)
NR328	53.8 (100)	20.2 (38)	0.9 (2)
NR345	87.2 (100)	36.2 (42)	4.5 (5)
Williams	53.1 (100)	28.3 (53)	4.5 (8)
nr_1	66.4 (100)	22.3 (42)	5.4 (10)

[a] data in brackets are expressed as a percentage of the N-free controls

However, the genotypes did not differ in sensitivity to combined nitrogen (calculated $F_{interaction} = 1.74 < F_{8,67}(0.05) \simeq 2.15$). This can be seen more clearly when the data for each genotype were expressed as a percentage of N-free controls (Table 9.5). Urea-grown plants in these experiments had considerable NR activity without nitrate being added to the assay solution (data not shown). Evidently, in this sand culture, considerable nitrification of urea had occurred. Nevertheless, it is clear that cNR-deficiency did not confer nitrate-tolerant nodulation in nr_1, NR328 or NR345 plants.

9.3.4 Plant fresh weight accumulation in N_2-dependent and nitrate-grown plants.

Table 9.6 shows plant fresh weights of Bragg, NR328 and NR345 plants after 7 weeks culture on N-free or KNO_3-supplemented nutrients. In the absence of nitrate, differences between Bragg and the mutants may have been due to decreased vigour in the mutant lines or due to inferior seed quality in the mutants. However, these differences were not significant. This experiment does need to be repeated, however, the salient feature illustrated in Table 9.6 is that nitrate significantly stimulated plant fresh weight accumulation in Bragg and the two mutants. Two-way analysis of variance showed that the effect of nitrate was highly significant (calculated $F = 105.8 > F_{1,30}(0.05) = 4.17$), but that the genotypes were not significantly different and that they did not respond differently to nitrate being supplied.

Table 9.6: Effect of nitrate on plant fresh weight accumulation in Bragg and M_4 progeny of NR328 and NR345. Plants were inoculated with *R. japonicum* strain CB1809 and cultured and harvested as described in Table 9.4. Each entry in the table is the mean of six plants. Two-way analysis of variance on the data indicated that the effect of nitrate-supplementation was significant, but that there was no difference between the genotypes.

Table 9.6

Genotype	Plant fresh weight (g)	
	N-free	2.5mM KNO_3
Bragg	16.2	45.7
NR328	8.2	43.4
NR345	11.6	34.7

9.4 Discussion

The screening approach used to isolate the mutants described in Chapters 6 and 10 entailed observation of the root system for altered nodulation phenotypes. The pertinent biochemical alterations in these developmental mutants are not known at this stage. The results presented in this chapter show that it is also possible to screen successfully for a specific enzyme activity. Two cNR mutants, NR328 and NR345, were isolated using an *in vivo* NR screen.

An *in vivo* assay procedure has also been used to isolate NR mutants in barley (Warner *et al.*, 1977) and pea (Kleinhofs *et al.*, 1978). In contrast to the approach used to identify NR328 and NR345, cNR mutant nr_1 (formerly LNR-2) was isolated using chlorate as a positive selection agent (Nelson *et al.*, 1983). The screening procedure described in this chapter could also be used to screen re-mutagenized cNR-deficient lines for iNR-deficiency. The direct assay for NR activity may have advantages over testing for chlorate-resistance, in view of possible secondary effects of chlorate (Cove, 1976).

NR328 and NR345 were recovered from separate M_2 families 328 and 345. Both of these families segregated for the mutant character, indicating that the mutation arose from the mutagenesis treatment and also that NR328 and NR345 are the resultant of separate mutation events. The two mutants were recovered from approximately 2,450 M_2 plants screened in EMS-population 2. No mutants were recovered from approximately 7,550 M_2 plants screened in EMS-population 1. The frequency of chlorophyll-

deficient mutants in population 1 and population 2 was 0.9% and 2.8%, respectively (Chapter 3). Thus, the frequency of *nts* (Chapter 6), cNR and chlorophyll-deficient mutants was consistently higher in population 2 than in population 1.

M_2 selections NR328 and NR345 were pure breeding and homozygous for the respective mutations and the stability of the mutant characters has been demonstrated through to the M_5 generation (Fig. 9.2). NR328 differed from NR345 and nr_1 in that it was leaky. Mutants nr_1 (Nelson *et al.*, 1983) and NR345 had negligible cNR activity, whereas NR328 had approximately 15% of the wild-type activity (Tables 9.1 and 9.2). Constitutive NR-deficient mutant nr_1 lacks both c_1NR and c_2NR activities that are present in parent cultivar Williams (Streit *et al.*, 1985). Studies on wild-type Bragg, grown in the absence of nitrate, have shown that this cultivar also expresses c_1NR and c_2NR activity (Mortimer, 1983). It therefore appears that NR345 described here also lacks both c_1NR and c_2NR activity. On the other hand, it is plausible that NR328 lacks either c_1NR or c_2NR activity or, alternatively, this mutant may have decreased amounts of both cNR activities.

Both NR328 and NR345 expressed iNR activity (Table 9.3). When NR activity was dependent on endogenous nitrate ($-NO_3^-$ assay), unifoliolate leaves of nitrate-grown Bragg, NR328 and NR345 plants had the same NR activity. The addition of nitrate to the assay medium resulted in Bragg having significantly higher NR activity than NR328 and NR345. It is likely, therefore, that Bragg had more NR enzyme (presumably cNR) present *in situ*, and that substrate supply limited activity in the $-NO_3$ assay. The presence of iNR in NR328 and NR345 was also reflected in the fact that nitrate stimulated plant fresh weight accumulation in

nitrate-grown NR328 and NR345 plants (Table 9.6). Thus, NR328 and NR345 are similar to nr_1, in that iNR was expressed and nitrate utilization was evident. However, in addition to being leaky, NR328 was also unique in that nitrate-grown plants developed necrotic lesions on the distal margin of mature leaves. This did not occur in N_2-dependent NR328 plants and thus the necrosis observed in nitrate-grown plants was probably due to nitrate accumulation. Necrosis of mature leaf margins was not a feature of nitrate-grown Bragg, NR345 or *nr*$_1$ plants and it is therefore possible that iNR activity is impaired at some stages of development in NR328. These hypotheses are currently being tested. Furthermore, allelism tests are being conducted on nr_1, NR328 and NR345. Crosses have also been made between Bragg and the mutants to determine the nature of inheritance of the NR328 and NR345 characters (D. Smith, *pers. comm.*).

Mutant nr_1 has been extensively characterized. The mutant character is inherited as a Mendelian monogenic recessive (Ryan *et al.*, 1983), but it is not clear whether the mutation is located in a regulatory gene or in a structural gene required for both c_1NR and c_2NR activities (Robin *et al.*, 1985). Mutant nr_1 also lacks *in vivo* $NO_{(x)}$ evolution normally present in the wild-type (Ryan *et al.*, 1983). The $NO_{(x)}$ compound evolved during an *in vivo* NR assay (Harper, 1981) has since been identified as being predominantly acetaldehyde oxime (Mulvaney and Hageman, 1984). All F_2 cNR-deficient segregants derived from Williams x nr_1 crosses also lacked acetaldehyde oxime evolution, and all cNR-positive segregants (i.e. wild-types) evolved acetaldehyde oxime (Ryan *et al.*, 1983). Furthermore, soybean root tissue (Harper *et al.*, 1985) and cell cultures (Nelson *et al.*, 1984)

lack both cNR activity and *in vivo* acetaldehyde oxime evolution. Although these genetic and physiological studies indicated that *in vivo* acetaldehyde oxime evolution is associated with cNR activity, the nature of this association has not been further characterized. NR328 and NR345 may contribute to the knowledge on acetaldehyde oxime evolution and its relationship to cNR activity.

As reported by Ryan *et al.* (1983), the nr_1 mutation did not impede nitrate utilization or confer nitrate-tolerance to nitrogen fixation. The results presented here also showed that cNR-deficiency in NR328, NR345 and nr_1 did not condition nitrate-tolerant nodulation in these lines. Both nodule number (Table 9.4) and nodule growth (Table 9.5) in the mutants were as sensitive as the wild-type to nitrate and urea inhibition. Although nitrogen fixation does not appear to be nitrate-tolerant in nr_1 (Ryan *et al.*, 1983), this area needs to be investigated in NR328 and NR345. The effect of nitrate on nodulation and nitrogen fixation has been studied in pea mutant E_1. Pea does not appear to have cNR activity (Lahav *et al.*, 1976) and E_1 is an iNR mutant possessing 20% of the wild-type *in vivo* NR activity (Feenstra *et al.*, 1982). Nitrogen fixation in E_1 appears to be nitrate-tolerant. In experiments by Feenstra *et al.* (1982), wild-type and E_1 plants were cultured with *R. leguminosarum* on N-free nutrients (i.e. they were N_2-dependent) and were then treated for two days with nitrate-containing nutrients. Nitrogenase (acetylene-reduction) activity in the wild-type was 47% inhibited by the nitrate treatment, whereas nitrogenase activity in mutant E_1 was only inhibited by 19%. These results indicated that either

carbohydrate deprivation or products derived from nitrate reduction, and not nitrate *per se*, was responsible for nitrate inhibition of specific nitrogenase activity in the wild-type (Feenstra *et al.*, 1982; Jacobsen, 1984). In contrast, nodule initiation and nodule development were inhibited to the same extent by nitrate in E_1 and the wild-type (Jacobsen, 1984). It was hypothesized that the nitrate concentration in the plant and/or in the growth medium suppressed nodulation (Jacobsen, 1984). However, a cautionary note that needs to be stated is that nitrate accumulated to toxic levels in E_1 and affected lant vigour (Feenstra and Jacobsen, 1980), and therefore the relevance of this data to explaining nitrate inhibition of nodulation in wild-type pea should be considered tentative. Also, the relevance of this data to nitrate inhibition in soybean is questionable (see Chapter 1). Recent studies on soybean have implied that the nitrate concentration in the growth medium, rather than the rate of nitrate uptake, is critical in nitrate-inhibition of the initial stages of nodulation (Harper and Gibson, 1984a; Gibson and Harper, 1985). However, in these experiments nitrate was administered as $NaNO_3$ and Na^+ controls were not included (A. Gibson, *pers. comm.*), and therefore this work requires clarification. Clearly, mutants altered in iNR activity or nitrate uptake would greatly facilitate the partitioning of nitrate effects on nodulation and nitrogen fixation in soybeans.

Although cNR constitutes approximately 12.5 to 20% of total NR activity in soybean (Harper *et al.*, 1985), the physiological significance of this form of NR remains obscure. The fact that nr_1 was selected on the basis of chlorate resistance

implies that cNR reduces nitrate *in planta* (assuming nitrate and chlorate behave the same in this respect) (Nelson *et al.*, 1983). However, cNR activity does not appear to be regulated by nitrate (Nelson *et al.*, 1983) or ammonia (Mortimer, 1983) and lack of cNR activity does not impede nitrate utilization (Ryan *et al.*, 1983). Further characterization of NR328 and NR345 may lead to a better understanding of the role of cNR in soybean.

CHAPTER TEN

ISOLATION OF NON-NODULATION MUTANTS *nod*49, *nod*772 AND *nod*139.

10.1 Introduction

Non-nodulation (or nod$^-$) mutants have been shown to occur in several legume species. These include *Trifolium pratense* (Nutman, 1946; Nutman, 1981), *Pisum sativum* (Lie, 1971; Holl, 1975; Ohlendorf, 1983a; Kneen and La Rue, 1984), *Medicago sativa* (Peterson and Barnes, 1981), *Arachis hypogea* (Gorbet and Burton, 1979; Nambiar *et al.*, 1983) and *Melilotus alba* (T.A. La Rue, *pers. comm.*). Prior to this study, only one non-nodulation mutant has been reported in soybean (Williams and Lynch, 1954). This naturally-occurring mutation, called rj_1, is inherited as a Mendelian recessive (Williams and Lynch, 1954). Although the rj_1 mutation conditions non-nodulation, the blockage in nodulation can be partly circumvented by inoculation with high *Rhizobium japonicum* cell numbers (La Favre and Eaglesham, 1984). The precise blockage point in symbiotic development in rj_1 rj_1 soybeans has not been determined, and this is also the case for non-nodulation mutants reported in other legume species.

This chapter describes the isolation of three non-nodulating mutants from mutagenized soybean populations. The three mutants have been designated *nod*49, *nod*772 and *nod*139.

10.2 Materials and Methods

10.2.1 Field screening. Approximately 20,000 M_2 seedlings were screened for non-nodulation in the field. The seedlings were derived from a mixture of seven M_2 populations. These populations were GR15, GR20, GR25 (derived from gamma-ray mutagenesis), NaN_3-1, NaN_3-2, NaN_3-3 (derived from NaN_3 mutagenesis) and EMS-population 1 (derived from EMS mutagenesis) (Chapter 3). The seeds were planted in the field (nitrogen content was low in the soil) and inoculated with peat cultures of *R. japonicum* strain CB1809. The plants were watered as required by spray irrigation, and at six to eight weeks after sowing the plants were up-rooted and screened for nodulation.

10.2.2 Glasshouse screening. The plant material screened for nodulation was EMS-derived M_2 families; 326 families were derived from EMS-population 1 and 560 families were from EMS-population 2. Ten seeds per M_2 family in population 1 were planted, whereas five seeds were planted per M_2 family in population 2. In total, approximately 5,200 M_2 plants were screened for non-nodulation. The seeds were planted in sand trays (63cm long, 23cm wide and 6.5cm deep). The trays were inoculated with peat cultures of *R. japonicum* strain CB1809 and were watered with N-free nutrients as described in Chapter 9. Incandescent bulbs supplemented natural light such that the photoperiod was 16h and glasshouse temperatures were maintained between 14°C and 30°C. The plants were screened for nodulation five to eight weeks after planting.

10.2.3 Stability of non-nodulating mutants. M_2 selections were grown through to seed and resulting M_3 progeny were tested in the glass-house for nodulation on N-free nutrients. Mutant and parental lines were cultured in either Leonard jars (Gibson, 1980) or 15cm diameter pots of river sand. In Leonard jar experiments, seeds were surface-sterilized by rinsing in 95% ethanol followed by 7 to 10 min in 3% sodium hypochlorite. After several rinses in sterile distilled water, the seeds were placed on water agar plates and allowed to germinate at 28°C in the dark. Two to three days later, when the radical was 0.5 to 1cm long, the seedlings were planted in Leonard jars. The jars remained un-inoculated or were inoculated with *R. japonicum* strains CB1809, USDA201 or USDA257 (the latter two strains are fast-growing Chinese isolates obtained from Dr. B. Rolfe, ANU, Canberra). In sand pot experiments, the plants were inoculated with *R. japonicum* strain CB1809 and watered with N-free nutrients as described in Chapter 9.

10.3 Results

Three stable non-nodulating mutants were isolated. Mutant *nod*49 came from the mixed M_2 population screened in the field. The M_2 population was derived from gamma-ray, NaN_3 and EMS mutagenesis. All M_3 progeny derived from M_2 selection *nod*49 failed to nodulate. The stability of this mutant character was also verified in the M_4 generation (Table 10.1). In Leonard jar experiments, *R. japonicum* strains CB1809 and USDA257 nodulated the parent cultivar Bragg, whereas *R. japonicum* strain USDA201 failed to nodulate Bragg (Table 10.1). In contrast, none of these strains nodulated *nod*49 (Table 10.1).

Mutant *nod*772 was isolated in the glasshouse from M_2 family 772, which belonged to EMS-population 1. Two out of nine plants tested in this family were nod^-. One of these nod^- selections produced M_3 progeny and all ten M_3 plants failed to nodulate in sand pots when inoculated with *R. japonicum* strain CB1809. The stability of the *nod*772 character was also confirmed in the M_4 generation with CB1809 as the inoculant strain (Table 10.2). Fifty days after planting Bragg plants had on average 22 nodules, whereas one *nod*772 plant had two small nodules and the remaining four *nod*772 plants tested did not nodulate (Table 10.2).

Mutant *nod*139 was isolated from M_2 family 139, which belonged to EMS-population 2. In the original screen, one out of five plants in this family did not nodulate. This nod^- selection failed to produce seed, but the mutant line was recovered by screening the remaining plants in M_2 family 139.

Table 10.1: Effect of inoculant strain on nodulation of Bragg and *nod*49 (M_4 generation). Plants were cultured in Leonard jars with N-free nutrients. The plants were harvested 40 days after planting. Each entry in the table is the mean $\pm$ S.D. of two or three plants.

Table 10.1

Soybean line	*R. japonicum* strain	nodule number . plant^{-1}	nodule fresh wt (mg). plant^{-1}
Bragg	uninoc	0	0
Bragg	CB1809	14 ± 2	218 ± 67[a]
Bragg	USDA201	0	0
Bragg	USDA257	52 ± 6	164 ± 11[a]
*nod*49	uninoc	0	0
*nod*49	CB1809	0	0
*nod*49	USDA201	0	0
*nod*49	USDA257	0	0

[a]Bragg nodules induced by *R. japonicum* strains CB1809 and USDA257 were effective.

Table 10.2: Nodulation of Bragg, *nod*772 (M_4 progeny) and *nod*139 (M_3 progeny). Plants were inoculated with *R. japonicum* strain CB1809 and cultured on N-free nutrients in 15cm diameter pots of river sand. The plants were harvested 50 days after planting. Each entry in the table is the mean (± S.D.) of 5 to 12 plants.

Table 10.2

Soybean line	nodule number . plant^{-1}	nodule fresh weight (mg) . g plant fresh weight^{-1}
Bragg	22.0 ± 12.8[a]	30.6 ± 9.9[a]
*nod*772 (M_4)	0.4[b]	0
*nod*139 (M_3)	0[c]	0

[a] mean ± S.D. of seven plants

[b] mean of five plants; four plants were nod$^-$ and one plant had two very small nodules

[c] all 12 M_3 plants of *nod*139 did not nodulate.

Three out of 46 plants in the secondary screen were nod$^-$. All M_3 progeny derived from one of these nod$^-$ selections failed to nodulate with *R. japonicum* CB1809 present, whereas wild-type Bragg plants did nodulate (Table 10.2).

10.4 Discussion

The results presented here show that non-nodulating mutants can be isolated from mutagenized soybean populations. The three mutants recovered were homozygous for the respective mutations. The stability of the mutant characters in *nod*49, *nod*772 and *nod*139 has been demonstrated through to the M_5, M_4 and M_3 generations, respectively.

It is not certain that the mutation(s) in *nod*49 resulted from the mutagenesis conducted in this study (Chapter 3). However, the fact that M_2 families 772 and 139 segregated for the respective mutant characters indicates that these mutations were EMS-induced. Furthermore, the mutations in *nod*772 and *nod*139 must have arisen from separate mutation events. Allelism tests are being performed on the three mutants isolated here and the naturally-occurring rj_1 mutant. The three non-nodulating lines have also been crossed with Bragg to ascertain the mode of inheritance of the mutant characters (A. Mathews, *pers. comm.*).

Mutant *nod*49 has been studied in greater detail than the other two mutants. As shown in Table 10.1, two strains that were able to nodulate parent cultivar Bragg did not nodulate *nod*49. Subsequent studies have shown that *nod*49 is resistant to many strains of *R. japonicum* that normally nodulate Bragg; however, high dose inoculation with some strains resulted in a few nodules being formed on mutant plants (A. Mathews, *pers. comm.*). Similar results have been reported for non-nodulating rj_1 soybeans (La Favre and Eaglesham, 1984). The physiological basis of this phenomenon is not known.

Prior to the formation of visible nodules, several processes occur in legume-*Rhizobium* associations. These include colonization and attachment of *Rhizobium* to the root, infection thread formation and cortical cell differentiation (Rolfe *et al.*, 1981; Bauer, 1981; Calvert *et al.*, 1984b). Mutant *nod*49 does not have cortical cell proliferation sites (W.D. Bauer, *pers. comm.*). that are characteristic of wild-type soybeans inoculated with an infective strain of *R. japonicum* (Calvert *et al.*, 1984b). It is not known whether the lesion in *nod*49 directly affects cortical cell division or another early step in host-*Rhizobium* interaction. Mutant plants did have slightly less lateral roots (Carroll, Mathews and Gresshoff, unpublished data), indicating that root differentiation may be affected in *nod*49. Nambiar *et al.* (1983) reported that a non-nodulation trait in groundnut was associated with the absence of root hairs. In contrast to this line, however, root hair development does not appear to be affected in *nod*49.

Extensive characterization of the mutants described here should provide valuable information for identifying the critical processes involved in nodule formation. In the agronomic context, these mutants are also potentially useful. In a large proportion of the United States soybean production area, symbiotically-inferior indigenous strains of serogroup 123 out-compete superior introduced strains for nodule occupancy (Moawad *et al.*, 1984). Circumvention of the problem of competition has been proposed by genetic means, entailing engineering a host with restricted specificity, such that symbiotically-inferior strains are excluded from entry into nodules (Devine and Weber, 1977). The first step in the development of a symbiosis with restricted specificity is the isolation of non-nodulating mutants. The second phase involves

identifying strains that suppress the non-nodulation trait in the mutant host. Attempts to identify *Rhizobium* strains that completely suppress non-nodulation in rj_1 soybeans (Devine and Weber, 1977; La Favre and Eaglesham, 1984) and *nod*49 (A. Mathews, *pers. comm.*) have not been successful. However, a preliminary experiment has indicated that the non-nodulation character in *nod*772 may be strain-specific (McNeil, Carroll and Gresshoff, unpublished data). This characteristic of *nod*772 is currently being verified. The non-nodulation mutants described here may also be useful in estimating nitrogen fixation in isogenic wild-type soybeans. The rj_1 mutation has been a useful tool in this regard (Weber, 1966); however, transfer of the character to another cultivar such as Bragg would require repeated back-crossing and selection to purify the genetic background of the rj_1 mutation.

CHAPTER ELEVEN

GENERAL DISCUSSION

The legume root nodule symbiosis may be viewed as a controlled disease (Rolfe and Shine, 1984) since it involves the interaction of the legume host and the invading *Rhizobium*. The focus of this research has been to assess the contribution of the plant to the regulation of the soybean - *R. japonicum* symbiosis, and particular emphasis was given to the controlling influence of nitrate.

The carbohydrate deprivation hypothesis has been proposed to explain nitrate effects on nodulation and nitrogen fixation (Oghoghorie and Pate, 1971). However, in nitrate-grown soybean plants there is no evidence of bacteroid (Chapter 4) or nodule (Streeter, 1981) carbohydrate deprivation and data presented in Chapter 5 indicate that O_2 supply to the nodule is a major factor limiting nitrogenase activity. Inhibition of specific nitrogenase activity in plants that were grown continuously on high nitrate could be alleviated by raising the atmospheric O_2 concentration during the acetylene reduction assay. Similarly, O_2 limitation was also evident during the early stages of nitrate- and dark-induced nodule senescence.

In the later stages of nodule senescence, differences were discernible in isolated bacteroids from nitrate- and dark-treated plants. Bacteroid integrity was eventually affected in nitrate-treated plants, whereas a depletion in endogenous energy reserves capable of supporting nitrogenase activity was evident in bacteroids from dark-treated plants. The quantities of leghemoglobin and other proteins also eventually declined during nitrate- and dark-induced nodule senescence (Chapter 4; Pfieffer *et al.*, 1983; Schuller *et al.*, 1985). However, O_2 limitation appeared to precede other detectable changes that could plausibly affect nodule activity.

It has been reported that O_2 supply to the nodule limits nitrogenase activity in water-stressed soybean plants (Pankhurst and Sprent, 1975a). This report, together with the results in Chapter 5, strongly imply that O_2 limitation in the nodule is a common control mechanism for regulating nitrogenase activity in symbiotically-stressed soybeans. With regard to nitrate inhibition, it is clear that the carbohydrate deprivation hypothesis requires at least some modification. Sugar levels are reported to be lower in soybean leaves during culture on nitrate (Streeter, 1981) and this may be the stimulus for the O_2 limitation response in the nodule. Similarly, both water stress and darkness reduce photosynthesis and affect the carbohydrate status of the shoot (Huang *et al.*, 1975; Schweitzer and Harper, 1980). The plausible mechanisms of O_2 deprivation within the nodule were alluded to in Chapter 5, as was the possibility that the mechanisms may not be common among the various stress conditions. This is an obvious area for future research.

Although with some reservation (Chapter 9), nitrate inhibition of the early stages of nodulation appears to occur prior to the reduction of nitrate in the host tissue. Nitrate reductase-deficient pea mutant E_1 is nitrate-tolerant for nitrogen fixation (Feenstra *et al.*, 1982) but not for nodulation (Jacobsen, 1984). Similarly, the exogenous nitrate level rather than nitrate uptake appears to be important in inhibiting the early stages of nodulation in soybean (Gibson and Harper, 1985). In this thesis, several *nts* mutants of soybean are reported that have increased nodulation in the presence of nitrate (Chapters 6 and 7). Mutant line *nts*382 was investigated in the greatest detail and was found to be similar to the pea mutant nod_3 (Jacobsen and Feenstra, 1984; Jacobsen, 1984).

As mentioned previously (Chapters 1, 6 and 7), the number of nodules produced on wild-type legumes is tightly regulated. The rapid regulatory response controlling the extent of nodulation (Pierce and Bauer, 1983) appears to be anomalous in *nts*382 and nodulation occurs at a high density over most of the root system regardless of the presence or absence of combined nitrogen (Chapter 7). Nitrate stimulates growth in *nts*382 and this mutant has normal nitrate reductase activity (Chapter 6). Thus, *nts*382 is a mutant in the regulation of nodule development and the term supernodulator is used to describe this line. It is now known that all of the *nts* mutants described in detail in Chapter 6 are also mutants in the regulation of nodule development and not in nitrate metabolism (Carroll and Gresshoff, unpublished data). These supernodulating mutants are also nitrate-tolerant for nodulation (Chapters 6 and 7; Carroll and Gresshoff, unpublished data) and therefore it is likely that the mechanism of nitrate inhibition of nodulation is mediated through the regulatory mechanism that is altered in the *nts* mutants.

As a result of increased nodule mass, *nts* plants had increased nitrogenase (acetylene reduction) activity in the presence of nitrate (Chapters 6, 7 and 8). Specific nitrogenase activity was, however, consistently lower in the *nts* mutants (Chapters 6 and 8). Interestingly, the presence of nitrate did not further inhibit specific nitrogenase activity *nts*382 (Chapter 6). It is not known at this stage whether 0_2 deprivation plays a role in the low specific nitrogenase activity measured in the *nts* mutants.

Under glasshouse conditions, excessive nodulation in *nts*382, *nts*1007 and *nts*183 was associated with decreased growth (plant fresh weight accumulation). However, some of the *nts* mutants have a

less extreme phenotype and poor growth may not be an intrinsic property of these lines. Furthermore, the extreme phenotype observed in the glasshouse may not occur to the full extent in the field and preliminary data indicates that this is the case (D. Herridge, *pers. comm.*).

Another feature of *nts*382 is that it has increased lateral root formation in the early stages of development (Carroll, Day and Gresshoff, unpublished data). This characteristic suggests that growth regulators may be involved in the supernodulation phenotype. It has been recently shown that foliar or soil applications of growth regulators can marginally influence nodule number on wild-type soybeans (Williams and Sicardi de Mallorca, 1984).

Nitrate inhibited nodulation in the three constitutive nitrate reductase (cNR)-deficient mutants nr_1, NR328 and NR345. Mutant nr_1 (formerly LNR-2) was isolated from a chlorate-resistance screen by Nelson *et al.* (1983), whereas NR328 and NR345 were identified in this study using an *in vivo* screen for cNR activity (Chapter 9). Nitrogen fixation appears to be sensitive to nitrate in nr_1 (Ryan *et al.*, 1983), but it is not known at this stage whether nitrate inhibits specific nitrogenase activity in NR328 and NR345. Whilst all three mutants possess inducible NR activity (Nelson *et al.*, 1983; Chapter 9), NR328 is unique in that it is leaky for cNR activity and it also eventually develops leaf necrosis when grown on nitrate. Inducible-NR pea mutant E_1 also develops leaf necrosis with culture on nitrate (Feenstra and Jacobsen, 1980). Clearly, NR328 and NR345 are potentially useful for studying the multiple nitrate reductases and related aspects of nitrogen metabolism in soybean (Harper *et al.*, 1985; Chapter 9).

Most of the mutant selections in this investigation were carried out on M_2 families (Chapters 6, 9 and 10). Although harvesting M_2 seed from each individual M_1 plant was more laborious than a bulk harvest, it did have salient advantages. It is almost certain that all the mutants, with the exception of *nod*49 (Chapter 10), came from independent mutation events. Harvesting the M_2 seed as individual families also allowed the mode of inheritance of the mutant characters to be tentatively ascertained without traditional genetic crossing. Furthermore, the segregation ratios in M_2 and M_3 families made it possible to estimate the genetically effective cell number (GECN) for soybean (Chapter 8). The inheritance data was consistent with the hypothesis that the *nts* characters are the result of monogenic mutations. However, soybean is considered to be a diploidized tetraploid (Lackey, 1981; Crane *et al.*, 1982) and the presence of multiple leghemoglobins (Verma and Nadler, 1984) and nitrate reductases (Harper *et al.*, 1985) may be indicators of ancient polyploidy in this legume species. Thus, the precise nature of inheritance of the mutant characters needs to be confirmed using traditional crossing methods.

The results reported in this investigation show that induced mutagenesis and large scale selection can yield valuable material. Analogous pea mutants have been isolated from EMS-derived M_2 populations (Jacobsen, 1984) and thus the prospect of isolating similar mutants in other legume species by the techniques outlined in this thesis is promising. The *nts*, cNR and non-nodulating mutants should contribute substantially more to the understanding of nitrogen metabolism in soybean. Mutants *nod*49, *nod*772 and *nod*139 should be useful in fragmenting the complex host-*Rhizobium* interactions that occur prior to nodule formation, and they may also be an avenue for overcoming

the problem of competition in the field, provided *Rhizobium* strains can be identified that suppress the non-nodulation phenotype (Devine and Weber, 1977; Chapter 10). The potential agronomic value of nitrate-tolerant symbioses has been described recently (Tudge, 1984). The possible advantages include increased residual nitrogen and improved soil structure following the legume crop, enhanced establishment of introduced inoculant strains, decreased need for nitrate fertilization and improved nitrogen status of the legume crop. The large set of independent *nts* mutants described here should confirm or deny these possible benefits.

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